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An Application of the Electric Flicker Method to Tests of Physical Strength in Normals and Tuberculous Patients

By

Makoto Kobayashi

(小林　真)

From the Physiological Laboratory of Prof. K. Motokawa
Tohoku University, Sendai

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Intdoruction

It has long been known that a subjective sensation of light, electrical phosphene, can be aroused by an electric stimulus passing through the eye. With this flickering phosphene as an index, Motokawa and his coworkers[1] determined the threshold strengths for alternating currents of varying frequencies. Motokawa and Iwama[1] found a striking effect of oxygen lack upon this phenomenon, and further Motokwawa and Suzuki[2)-5)] reported that the electric flicker could be used as a very convenient indicator for measurements of fatigue.

Recently this method has been applied not only to the industrial hygiene, but also to the field of clinical medicine[6)-8)]. In patients suffering from silicosis Nakamura *et al.*[9)-10)] found that certain exercise greatly increased the electric flicker value or ΔS, and measured the time necessary for increasing the value at twice its initial value. The time so measured was found to serve for specification of the physical status of the patients.

Examination of the physical strength in tuberculous patients is important not only for making an appropriate therapy but also for determinating when they should resume their work.

This subject has been investigated by various workers with various methods; Kunishima, Abeno, Hirai and Sashida[11)-13)] and Yamada[14)] and Yuzawa *et al.*[15)] and others investigated the fatigue in tuberculous patients. In this connection the work by Nakamura *et al.*[9)-10)] mentioned above is especially worth noticing, because the load time they measured represented the physical status of their patients very satisfactorily.

It seems, however, that there are some points to be improved in their method, because too severe exercise was required to cause an increase ΔS to twice the initial value. The circumstance that a recording apparatus

of ΔS is necessary for this method, is another shortcoming.

Therefore, it was attempted in the present investigation to reduce the trouble by measuring the time necessary to cause a least perceptible increase of ΔS, instead of an increase to twice the initial value. The method was applied to 43 healthy males, 37 healthy females and 210 patients suffering from pulmonary tuberculosis in the present investigation.

Experimental

Method

The method of electric flicker is described in detail in a previous paper[1)2)6)7)], so that only the point modified will be mentioned here.

At first, the mean of 10 ΔS values was obtained before a load exercise. The exercise consisted of a step test with a footstool which had two steps each 20 cm. in height. The subject repeated movements up and down the stool every 5 seconds. The load time was 8–10 minutes for normal subjects, but 3–6 minutes for tuberculous patients. They were, however, told that they might give up exercise whenever they would. It is characteristic of the present method that a desired value of test can be obtained whatever the actual load time may be. The mean of 10 ΔS was obtained again immediately after the load exercise and the standard deviation (S.D.) was also calculated from this second series of measurements of ΔS.

In Fig. 1, in which mean values of ΔS are plotted against load times, *a*

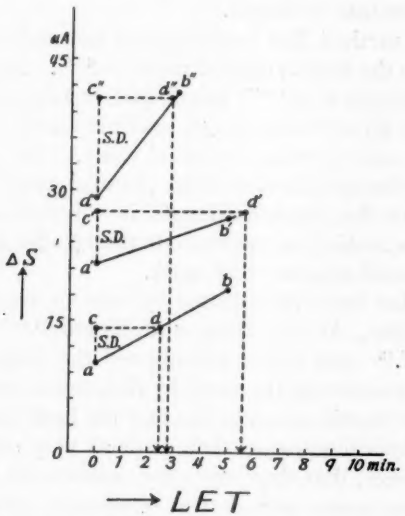

Fig. 1. Diagrams used for measuring LETs. Explanation in text.

and b represent the mean values of ΔS before and after the exercise respectively. Similar pairs obtained from other cases are represented by a' and b' and by a'' and b''. Vertical lines ac, $a'c'$ and $a''c''$ represent S.D.s obtained from respective cases. d,d' and d'' represent points of intersection between horizontal line cd and line ab, between $c'd'$ and $a'b'$, and between $c''d''$ and $a''b''$, respectively. Times corresponding to d, d' and d'' are measured, which will be denoted by " Least Effective Times " or LETs.

LETs are load times which would be required to increase the value of ΔS by its S.D. when it is assumed that ΔS increases linearly with load time. The longer the LET, the greater the physical strength.

The normal subjects tested were 43 healthy males of 20 to 53 years in our laboratory, and 37 healthy females of 18 to 27 years of age who were mostly nurses, the averaged age being 26.5 years. The tuberculous patients investigated were 210 adults treated as inpatients or outpatients at 6 hospitals in the city of Sendai. They consisted of 161 males of 16 to 60 years of age and 49 females of 13 to 57 years of age, the averaged age being 33.2 years.

1) LETs of normal subjects

The results obtained are illustrated in Fig. 2, in which diagrams (A) and (B) represent respectively a histogram and a distribution curve of LETs for normals. In these diagrams LETs are expressed in logarithmic scale, because the distribution is more symmetrical in this expression than otherwise.

As can be seen in the figure, there is no appreciable difference between male and female.

In Table I daily variations of LETs of three normal subjects are shown. As can be seen in this table, daily variations are not so marked. Values of LETs taken in the morning and in the afternoon on the 10th day show no significant difference.

2) LETs of tuberculous patients

In Fig. 2(C) distribution curves obtained from tuberculous patients are shown in comparison with the normal control (the curve marked by open circles). The continuous curve marked by dots refers to 210 patients, and the broken one marked by crosses to 15 patients who had so recovered that were going to resume their work. There are significant differences among these three groups.

Fig. 2(D) represents distribution curves of LETs for three different groups, namely male outpatients (open circles), male inpatients (crosses) and female inpatients (dots). LETs of the male outpatients are generally longer than those of the inpatients.

(A) The relation between LETs and " the degree of rest cure "

In national hospitals of Japan, tuberculous patients are classified into

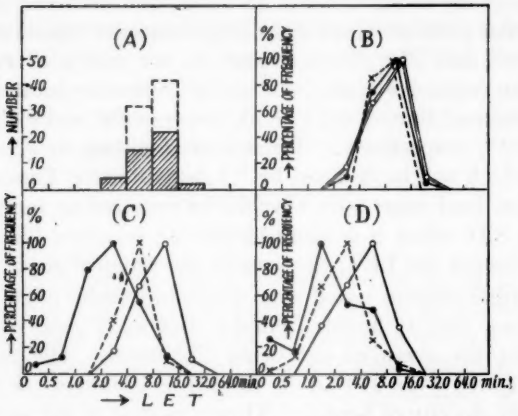

Fig. 2. Histograms and distribution curves of LETs obtained from normals and tuberculous patients. LETs are expressed in logarithmic scale.

(A) Histogram of LET obtained from 80 healthy adults. Rectangles drawn in full line and broken line refer to males and females, respectively.

(B) Distribution curves of LETs of normals. Curves marked by solid circles, by crosses and by empty circles refer to males, females and all together respectively.

(C) Distribution curves of LET obtained from normal adults, from patients just before resumption of office and from other patients marked by empty circles, by crosses and by solid circles respectively.

(D) Curves marked by solid circles, crosses and empty circles were obtained from male outpatients, male inpatients and female inpatients respectively.

8 classes according to the degree of rest necessary for each patient. The degree of rest is determined on the basis of various clinical examinations. The eight classes are labeled 1 to 8 from the most serious to the slightest one and this order is called " the degree of rest cure ". The LET-test was applied to patients with various degrees of rest cure. The curve marked by dots in Fig. 3 (A) shows the relation between LETs and degrees of rest cure. The curve is almost linear, indicating a close correlation between them. It is to be noted that the degree of rest cure, which is determined by various clinical factors, is represented very satisfactorily by a single measure, such as LET. As indicated by curves marked by solid triangles and by open circles, neither ΔS values nor standard deviation measured immediately after the load exercise show any simple relation to the degree of rest cure.

(B) The relation between the LET and the time elapsed since a chest operation

In a number of patients who received a chest operation some months

TABLE I

Daily Variations of LET in Minutes

Day \ Subjects		S.K.	M.K.	J.U.
1		10.8	9.0	7.5
2		10.0	9.5	7.0
3		8.7	7.5	
4		14.4	9.5	5.0
5		11.0	7.5	5.5
6		12.0	8.0	6.0
7		9.0	10.0	
8		9.0	12.5	7.9
9			8.3	6.9
10	a.m.	9.0	11.3	6·5
	p.m.	12.0	9.6	5.3
Mean		9.55	9.33	6.40
S.D.		1.43	1.31	1.01

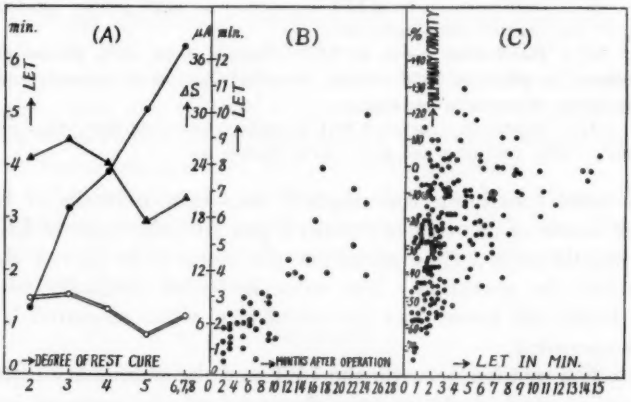

Fig. 3. Diagram (A) : Relations between degree of rest curve and LET (solid circles), ΔS (solid triangles) and S.D. of ΔS (empty circles).
Diagram (B) : Relation between LET and months after chest operation.
Diagram (C) : Relation between LET and pulmonary capacity.

before, LETs were measured to see correlation between the LET and the number of months elapsed. The results are shown in Fig. 3 (B), which indicates that the longer the time elapsed, the longer the LET. This finding indicates that the LET can serve as a good indicator for recovery.

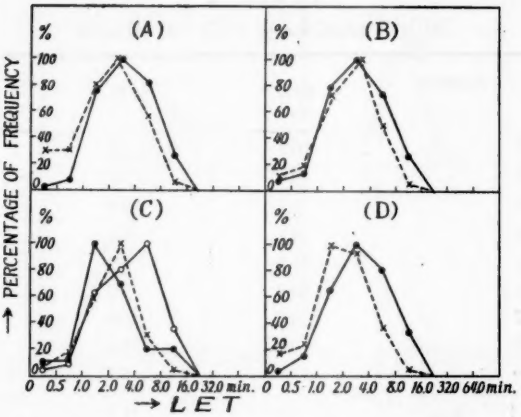

Fig. 4. Relation between LET and various kinds of clinical examination of 210 tuberculous patients.

(A) : Distribution curves of LET obtained from patients showing sedimentation rate below 10 mm/hr (solid circles) and from those showing rate above 10 mm/hr (crosses).

(B) : Distribution curves of LET in patients with bacilli positive (broken curve) and negative (continuous curve).

(C) : Distribution curves of LETs obtained from three groups of patients, far advanced (solid circles), moderately advanced (crosses), and minimally advanced (empty circles).

(D) : Distribution curves of LET in patients with pulmonary capacity below −30% (full line) and over −31% (broken line).

The same conclusion was reached from measurements of LETs in the entire course of recovery of certain 2 patients; the value of LET measured 2 months after a chest operation was found to be shorter than that found before the operation. The value increased gradually with time, but was found still lower than the initial level when measured 6 months after the operation.

(C) The relation between the LET and the pulmonary capacity

The percentage deviation from the standard vital capacity is defined as the pulmonary capacity. Negative values of it indicate decreases in vital capacity below normal. In Fig. 3 (C) pulmonary capacities of patients are plotted against corresponding values of LET. In general, there should be no simple relation between the pulmonary capacity and the LET, because the LET must be a function of various factors. In reality, the range of pulmonary capacity corresponding to any given value of LET is very wide, as can be seen in the figure, but it can not be overlooked that the minimal value of pulmonary capacity for each LET changes almost

linearly with LET.

This fact may be accounted for as follows: When the pulmonary capacity is impaired so severely that it becomes a determining factor for the physical strength of a patient, then this factor becomes to determine his LET, because the LET is an indicator of the physical strength.

(D) The relation between the LET and other clinical signs

Tuberculous patients were classified into two groups concerning blood sedimentation rates; one group showing rates below 10 mm per hour, and the other having rates above 10 mm per hour.

In Fig. 4 (A) the distribution curves of LET for these two groups are compared. The continuous curve for the group having lower sedimentation rates is shifted to the longer side of LET. The difference between the two curves was found statistically significant at a 1 per cent level.

Simillarly the patients were divided into two groups according to whether tuberculous bacilli were positive or negative in sputum or gastric juice, and their distribution curves of LET were compared in Fig. 4 (B). These curves were shown to be significantly different at a 5 per cent level.

The roentgenograms of the patient's chests were classified into 3 categories, that is, far advanced, moderately advanced and minimally advanced according to the method of by American Trudeau Society.[16] In Fig. 4 (C) the distribution curves of LET obtained from these 3 groups are shown. As will be seen in this figure, the curve for the far advanced group is situated on the left side, that for the minimally advanced group on the right side, and that for the moderately advanced group takes an intermediate position. Thus, values of LET correspond well to the severity of disease distinguished on the basis of roentgenograms.

Fig. 4 (D) refers to the distribution curves of LET obtained from 2 groups, showing pulmonary capacity less than -30% and that greater than -30%. The difference between the two curves was found statistically significant at a 5 per cent level.

Discussion

Individual differences of ΔS are generally so great that it is difficult to compare ΔS values obtained from different persons. Nakamura *et al.*[9][10] tried to avoid this difficulty, taking a load time necessary for increasing ΔS to twice its initial value as an index, instead of ΔS itself. The load times of healthy adults were found, on an average, about 33 minutes, while those of patients suffering from silicosis ranged from 2 to 18 minutes. Therefore, it was suggested that the method might be used for clinical examination of the physical status of patients. The exercise used in the present investigation was not so severe as that used by Nakamura *et al.*, and the load time was much shorter. This would be a great help in clinical ap-

plication. The measurement of LET is also very easy and practical.

As has been demonstrated in Fig. 3 (A), a close relation between the LET and the degree of rest cure can be found, while no such correlation can be found between the value of ΔS and the degree of rest cure. Some attempts were made in the present investigation to find any correlation between ΔS and other clinical signs such as blood sedimentation rates, and roentgenograms, etc., but no significant correlation could be found.

Similar attempts were made with other measures such as the standard deviation of ΔS, $\Delta S - \Delta S_0$, and $(\Delta S - \Delta S_0)/\Delta S_0$, where ΔS_0 means ΔS value before a load exercise, but all these attempts failed. So far as my investigation goes, the LET seems to be the only practical measure obtained by the method of electric flicker. I should like to recommend the application of the present method to similar but other kinds of examination.

Summary

Electrical thresholds for appearance and disappearance of electric flicker were measured, and the difference of the two values was denoted by ΔS. The values of ΔS were measured before and after a standard load exercise, a step test lasting several minutes.

A method was devised to find the time required for causing a least significant increase in ΔS by the standard load. The time so measured was designated a LET (least effective time).

LETs were measured in 80 healthy adults and 210 patients suffering from pulmonary tuberculosis.

1. In the experiment on normals, any sex difference could not be found concerning LET.
2. Daily variations of LET were not so significant.
3. The distribution curve of LETs for male inpatients occupied a position intermediate between that for male outpatients and that for female inpatients.
4. A high correlation could be found between LET and the degree of rest cure, the number of months after a chest operation, or the pulmonary capacity.
5. LETs showed close correlation to various clinical signs such as sedimentation rates of blood corpuscles, roentgenograms of the chest, etc.

It was concluded from these findings that the LET serves as an excellent indicator for recovery of the physical strength in patients.

I am greatly indebted to Prof. K. Motokawa for his generous guidance and valuable suggestions.

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Effect of Intraocular Pressure upon Electrical Excitability and Action Potentials of Human Retina

By

Tadashi Kohata, Masayoshi Wake and **Hironobu Hamada**
(木幡 正) (和気 正好) (浜田 宏信)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

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Introduction

Intraocular pressure is increased when mechanical pressure is applied to the eyeball, and this effect is utilized for determination of the arterial blood pressure at the retina.[1)-3)] Wake[4)] found that the electrical excitability of the retina is very sensitive to an increase of intraocular pressure and that the effect manifests itself in a lowering of electrical excitability before any sign of visual impairment appears.

As has been shown by Motokawa[5)6)], the electrical excitability of the dark-adapted eye increases temporarily above the resting level following a brief illumination and the time curve of this change is characteristic of the wave-length of the light used; the electrical excitability shows a maximum at about 1, 2 and 3 seconds respectively from the end of illumination when red, green and blue lights are used for illumination.

In the present experiment it was investigated how the color effect mentioned above was effected by an increase of intraocular pressure. In comparison, some experiments were carried out on the effect of raised intraocular pressure upon ERGs of one and the same subjects.

Experimental

Method

After a preliminary dark adaptation of about 20 minutes electrical thresholds of the eye were measured with single rectangular pulses of 0.1 sec. The detail of this procedure is described in previous papers by Motokawa[5-7)]. Electrical excitability measured under pressure was expressed in per cent of that obtained without pressure. The pressure was applied to the eyelids by means of a spring manometer devised by Wake.[4)] This apparatus consisted of a metalic cylinder provided with a spring. At one

end of the spring an eye-disc was attached, which was held in contact with the eyelids of the subject and served to transmit the pressure caused by the spring to the eyeball. The pressure was controlled by changing the length of the spring. This procedure could be done very easily by the subject.

Prior to each electrical test shock pressure of a given strength was applied to the eye and removed after the subject gave information about the electrical phosphene evoked by the electric stimulus. This procedure was repeated till a threshold was reached. In order to study the color effect, similar measurements were carried out with colored light.

The color effect was measured by the difference between the values of electrical excitability determined with and without pre-illumination. It was found that the color effect decreased as the pressure applied to the eyeball was increased and that it did not appear any more over a certain critical pressure. In our experiment pressure is given in terms of total pressure in grams at the eye-disc of the spring manometer. The disc was 25 mm. in diameter and had an opening at the center whose diameter was 18 mm. The subject viewed a target 2° in visual angle through the opening in the eye-disc. For the purpose of measuring the arterial blood pressure at the retina of our subjects other kinds of pressure apparatuses were constructed, because the spring manometer was not suitable for this purpose.

They are illustrated in Fig. 1 A and B.

The apparatus A consisted of a funnel, b, and variable weights, c. The lower end of the funnel (a) was held in contact with the eyelids of a subject lying on the back. The contact surface was the same as that of the spring manometer mentioned above. This apparatus was convenient for ophthalmoscopic examination of the retina, because the opening was sufficiently wide.

The other apparatus B was constructed following the principle of a sphygmomanometer. It consisted of a doughnut-shaped rubber bag, b, enclosed in an envelope of cotton cloth, c, to prevent it from stretching under pressure, and of two tubes, one connected to a manometer, e, and the other to a small hand pump, d. In this apparatus the eyedisc, a, was of the same size as that in the spring manometer mentioned above. With these two pressure apparatuses the arterial blood pressure at the retina was measured in these subjects, taking the disappearance of pulsation at the central retinal artery as an index. The results obtained are shown in comparison with similar values determined with Bailliart's dynamometer in Table I.

The great difference between the values obtained with our apparatuses and those obtained with Bailliart's dynamometer is due to the circumstance that pressure was applied indirectly through the eyelids to the eyeball with

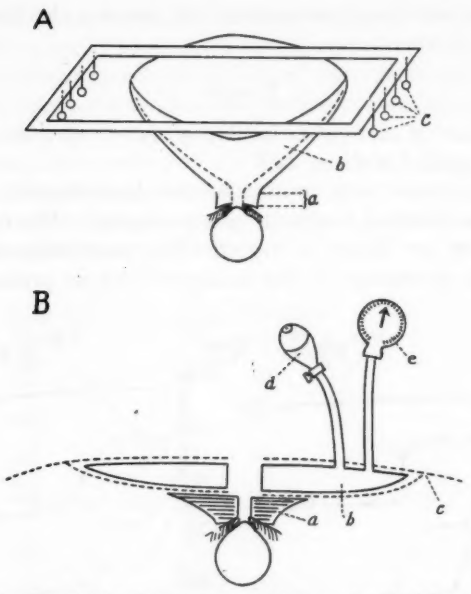

Fig. 1. Two kinds of pressure apparatuses. A: Used for ophthalmoscopic examination of retina (a, eye-disc; b, funnel; c, weights). B: Used for the same purpose as above (a, eye-disc; b, rubber bag; c, cotton cloth; d, hand pump; e, manometer).

TABLE I

Values of Arterial Blood Pressure at Human Retina

Subjects	Retinal blood pressure measured with		
	A of Fig. 1	B of Fig. 1	Bailliart's dynamometer
H.H.	480 g.	48 mm. Hg	85 g.
T.H.	480 g.	50 mm. Hg	85 g.
M.W.	470 g.	52 mm. Hg	—

our apparatuses, but directly with the dynamometer. There are also some differences between A and B. This may be due to the difference in the way of pressure transmission between the two apparatuses A and B.

In any case, the spring manometer which was used exclusively in the present experiment is nearest to the apparatus A in principle and construction. Therefore one may understand that a value of pressure about

480 g. caused by our spring manometer can just stop the blood flow at the central retinal artery.

Results

1. *Dependence of electrical excitability of the eye upon the duration of mechanical pressure applied to the eye ball*

A certain pressure was applied to the dark-adapted eye and after various times its electrical sensitivity was measured. The results obtained from two subjects are shown in Fig. 2. The excitability under pressure was expressed in percentage of that measured without pressure.

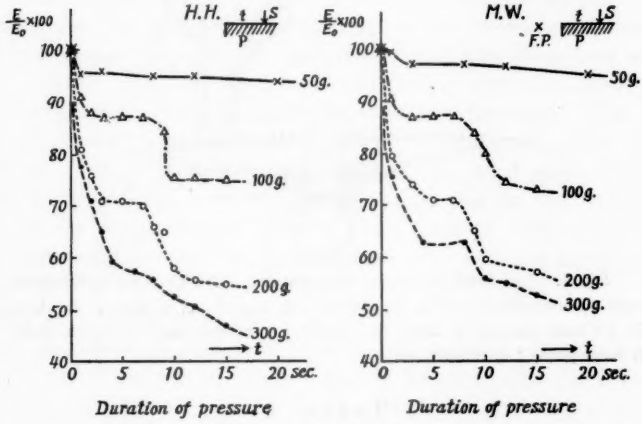

Fig. 2. Effect of pressure upon electrical excitability of eye as a function of duration of pressure. E and E_0 are excitabilities measured with and without pressure. In inset p, t and s denote pressure, duration of pressure and electric test stimulus respectively.

As can be seen in this figure, under pressure of 50 g. a lowering of several per cent takes place in 1 second and continues as long as the pressure endures. A similar initial decrease can be seen under other pressure conditions; the magnitude of decrease becomes greater and it takes longer time for reaching a stationary level as the pressure applied increases.

It is to be noted that for higher pressure than 50 g. a second steep decrease takes place at about 7 seconds from the onset of pressure. The value of excitability remains almost steady between 4 and 7 seconds and between 10 and 15 seconds. This property was utilized in the following experiments.

2. *Effect of white light upon electrical excitability and its dependence upon mechanical pressure*

White light causes temporary increase in electrical excitability which becomes highest at about 2 seconds from the end of the white light stimulus.

In the following experiment a flash of white light 10 lux in intensity and 40 msec. in duration was used, and an electrical test shock was delivered always at 2 seconds from the flash.

In one group of experiments the flash preceded the onset of pressure by 0.2 seconds; the electrical test shock was given at 2 seconds from the flash or at 1.8 seconds from the onset of pressure, which was removed after subject's response to the test stimulus.

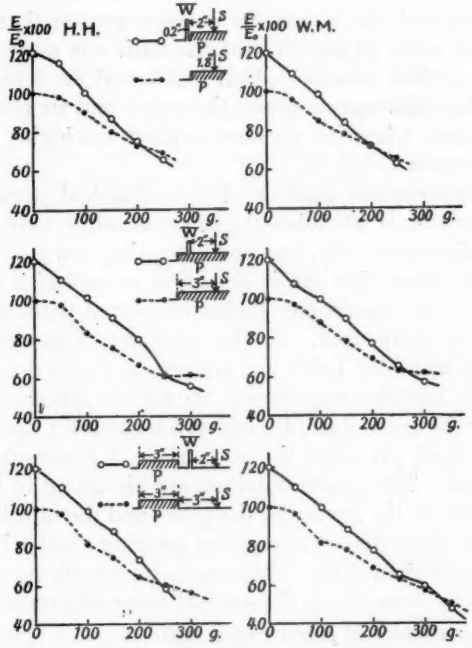

Fig. 3. Effect of pressure upon effect of white light. Continuous and broken curves refer to data obtained with and without illumination respectively. Effect of white light is represented by difference between two curves. In inset p, s and W denote pressure, electric test stimulus and a flash of white light respectively. Note temporal relation of W relative to p. Three sets of curves on left side were obtained from subject H. H., and as many sets on right side from subject W. M.

The uppermost set of curves in Fig. 3 represents the data obtained from 2 subjects. As a control, experiments were performed without the flash of white light, otherwise under one and the same experimental con-

ditions. The continuous and the broken curves refer to experiments with and without the flash respectively. The difference between the two curves becomes smaller as the pressure applied increases, and at about 200 g. the difference disappears. This finding indicates that the effect of the white light is abolished by pressure greater than 200 g.

In the second series of experiments the flash of white light was given 1 second after the onset of pressure which continued till the subject's response to the electric test shock. As can be seen in Fig. 3, the difference between the curve obtained under the action of the light and pressure together and the control curve obtained under pressure alone becomes smaller as the pressure increases and almost zero for pressure greater than 250 g.

In the third series of experiments the flash was given 1 second after removal of the applied pressure which had lasted for 3 seconds. As can be seen from the difference between the curve and its control, the effect of light disappeared when the pressure applied was higher than about 250 g. (the bottom curves in Fig. 3).

All these experiments indicate that mechanical pressure applied to the eyeball decreases or abolishes the effect of white light not only when they are given simultaneously, but also when they are given in succession.

3. *Effect of colored light and its dependence on mechanical pressure*

In the following experiments flashes of colored lights of equal energy, (green and blue) were used. As the increases of electrical excitability caused by green and blue lights are greatest at 2 and 3 seconds from the end of the photic stimulus respectively, the electric test shock was delivered at 2 seconds for the green flash, G, and at 3 seconds for the blue flash, B.

The green flash preceded the pressure by 0.2 seconds, and the blue flash by 1 second. The results obtained are illustrated in Fig. 4. As can be seen in this figure, the curves for the green and the blue lights are higher than the control curve determined under pressure alone when the pressure applied was lower than 200 g. However, no significant difference from the control can be seen above this critical value of pressure.

4. *Effect of mechanical pressure upon ERG*

As has been shown by the preceding experiments, the effects of white and colored lights as revealed by the method of electric stimulation are abolished by mechanical pressure as low as 200 g. This fact may be regarded as evidence that the effects are a retinal event, although a subjective index, electrical phosphene, is used for their measurements. It is unlikely that pressure of this order should abolish any cortical process, but it is very likely that a certain variation of retinal circulation should be caused by pressure of this order. The lowering of electrical excitability and the disappearance of the effect of light observed above must be due to some circulatory disorder in the retina. Granit[8] showed that the b-wave of

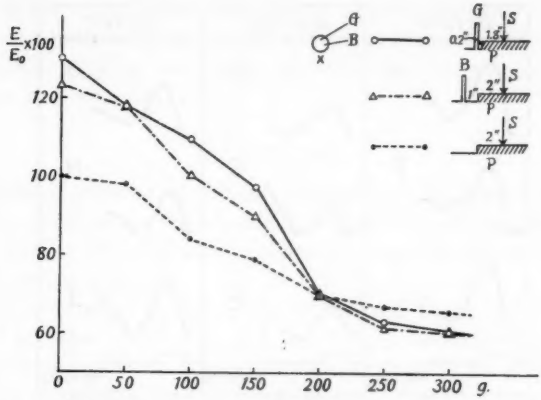

Fig. 4. Effect of pressure upon color effect. In inset B, G, p and s denote blue light, green light, pressure and electric test shock respectively. Color effect disappears at 200 g.

the ERG of the cat was considerably depressed when the carotid was occluded for 2 minutes. In order to see to what extent the ERG of our subjects would be suppressed by the mechanical pressure of the order of magnitude used in the present investigation, some experiments were carried out with the following method: A contact lens electrode applied to the cornea and an indifferent one, a silver plate 1 cm² in size placed on the glabella, were used for recording ERGs. The photic stimulus used was 10° in visual angle, 100 lux in intensity and 0.2 sec. in duration.

The cornea and the conjunctiva were anesthetized with 0.3 per cent percamin. Pressure was applied directly to the eyeball by means of the dynamometer. Some examples of ERGs are shown in Fig. 5. Records A, B and C were obtained 60, 30 and 5 seconds prior to the application of 30 g. They served as controls. Records D, E and F were obtained at 70, 140 and 160 seconds from the onset of pressure, and records G, H and I 60, 80 and 100 seconds after removal of the pressure respectively.

The level of resting potential showed so great variations shortly after the onset or removal of pressure that it was almost impossible to take records in these transitory periods.

At 30 g. and at still lower values of pressure the ERG, once reduced to a certain extent, remained almost constant for a considerably long time, but at higher values of pressure the ERG was progressively reduced with time. Pressure values 30 and 50 g. in the dynamometer were found equivalent to 130 and 230 g. respectively in our spring manometer. This was known from another series of measurements in which the effects of both

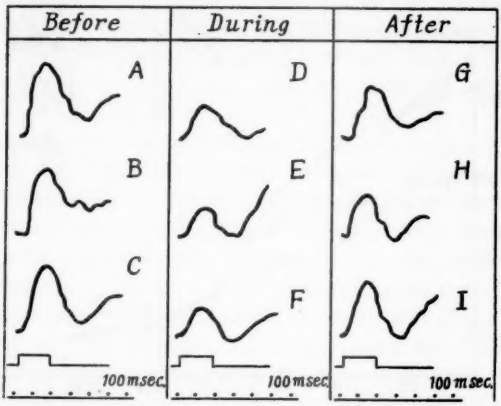

Fig. 5. Records of human ERGs taken before (A, B and C), during (D, and F) and after (G, H and I) application of pressure (30 g. by dynamometer).

apparatuses on the electrical excitability of the dark-adapted eye were compared.

Discussion

Changes of ERGs in local and general asphyxia have been investigated by several authors; Noell[9] recorded ERGs together with action potentials of the optic nerve in various mammals, and found that the asphyxial resistance of the retina showed great variations according to animal species. From comparison of the two phenomena he suggested that in the monkey, failure of conduction within the ganglion cell would be a decisive factor in determining the onset of blindness. Henkes[10] observed in a number of cases that the b-wave decreased following embolism of the central retinal artery and was restored greatly after several months. Motokawa & Iwama[11] studied the lowering of electrical sensitivity of the human retina caused by low atmospheric pressure in comparison with photosensitivity, and found that both kinds of sensitivity were affected almost in parallel. The effect of reduction of oxygen tension in breathing air upon the photosensitivity of the human retina was also investigated by Hecht *et al.*[12] and McFarland *et al.*[13]

In any case it is certain that high intraocular pressure affects the photo- and electrical sensitivity of the retina indirectly by way of local asphyxia in the retina, but not directly. As has been shown above, the effects of white light and colored lights are completely abolished by pressure of about 200 g. While the electrical excitability itself is not affected so severely. In Fig. 3 the level of electrical excitability, at which the total

abolition of the color effect took place, was 60 per cent of the normal excitability in both subjects. Such a low resistance of the color effect to local asphyxia suggests that some delicate nervous mechanism must be involved in this phenomenon.

Summary

Mechanical pressure of variable intensities was applied to the eyeball, to study its effect upon the electrical excitability of the human eye and upon the ERG. The electrical excitability was measured with electrical phosphenes as an index.

1. The electrical excitability was lowered by 5, 25, 45 and 55 per cent under pressure of 50, 100, 200 and 300 g., respectively in 15 seconds. The decrease caused by each pressure was not steadily progressive, but took place in two steps; the first decrease happened within 4 seconds and the second at 7 seconds from the onset of pressure.

2. It is known that the electrical excitability shows a temporary rise following an illumination. This effect of light was found to be abolished by pressure of about 200 g.

3. The time taken for the electrical excitability to reach a maximum is different according to the wave-length of the light used for illumination. This color effect disappeared under pressure of about 200 g.

4. In the experiments mentioned above, pressure was applied to a doughnut-shaped disc which was held in contact with the eyelids, the outer and the inner diameters of the disc being 25 mm. and 18 mm. respectively. Therefore it was necessary to compare the data obtained in this way with those obtained by other methods. Pressure values of 30 and 50 g. in Bailliart's dynamometer were found to be equivalent to 130 and 230 g. in our apparatus. The retinal arterial blood pressure was found 85 g. with the dynamometer, and 480 g. with our appratus.

5. ERGs of our subjects suffered from marked reduction under dynamometric pressure of 30 g.

6. From these findings it was concluded that the observed decrease in the electrical excitability of the eye and especially the abolition of the color effect were due to local asphyxia caused by pressure.

The writers are greatly indebted to Prof. K. Motokawa, under whose guidance this investigation was performed, for his generous advice and valuable suggestions throughout the course of the experiment and the preparation of the manuscript.

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A Study on the Specific Gravity of the Spinal Fluid

By

Kouki Ikeda
(池田 公喜)

From the Clinic of Internal Medicine of Prof. F. Nakazawa, Tohoku University, Sendai

(Received for publication, December 3, 1956)

Of the bodily humors, for blood and serum we have the excellent copper sulphate method for measuring their specific gravity, and the measurement is practically applied in many cases of diseases. For exsudative and transsudative fluids, which can be sampled in comparatively large volumes, the measurement is rather easy, so that the discrimination between them by specific gravity is possible, and to the anomaly in the specific gravity of urine is assigned a very high clinical significance.

But of the spinal fluid, which is subject to comparatively less marked influence of the external world than blood and exsudative fluid, in spite of its high importance for its function of mechanically protecting the central nerve system and its possible indispensability for the activity of the brain as its interstitial fluid, the specific gravity has not been recently taken up as a theme of discussion. One of the cause of such a neglect may lie in the inadequacy of the present knowledge on the subject, for the past estimations of the normal value of the specific gravity fluctuate within the wide range of 1.001 to 1.010, so that nothing can be stated with certainty on the difference between the normal and the pathological values.

With the hope of finding some significance in the findings to be obtained in this unexplored field of physiological information and if possible, of supplying a new means of service in diagnosis and treatment of some diseases, the author of this paper undertook the experiments reported on hereunder of measuring the specific gravity of spinal fluid, of which only a sparse quantity can be sampled in any case, by a new method perfected at this laboratory and accredited to yield excellent results of higher accuracy than any other available method, first for determining the specific gravity of normal spinal fluid and thence to follow up the changes of this value in the cases of pathological occurrences.

Experimental

Method of Measurement

The N-N Type (Nakazawa-Nakai Type) Dilution Hydrometer[1] was used, with an indicator ball of 1.0020 in specific gravity to water of 15°C sealed in. 2 cc. of spinal fluid was used per measurement.

The measurement was made at random temperature and then corrected to the value at 15°C by Nakai's formula of correction.[1]

Conditions of Measurement

Since the accuracy of $0.001 \times 1/10$ was to be maintained in this study, I had to pay attention to the following conditions among others. For measurements, always the same 2 cc. pipette and the same N-N hydrometer were used.

According to Nakai, Hayashi, Fujimoto *et* and *al.*,[2] a difference of 0.0001 from the density of the solution in equilibrium gives an acceleration of 0.38 mm/sec to the indicator ball, so that an ocular reading is possible. Thus, the indicator ball is sensitive enough for assuring the accuracy down to $0.001 \times 1/10$.

The difference in atmospheric pressure at the time of measurement can be entirely neglected. For avoiding the error due to the expansion of the indicator ball (though quite negligible in practice), a measurement under a temperature rather widely different from the standard temperature 15°C was abstained from.

Results

(Vide Fig. 1)

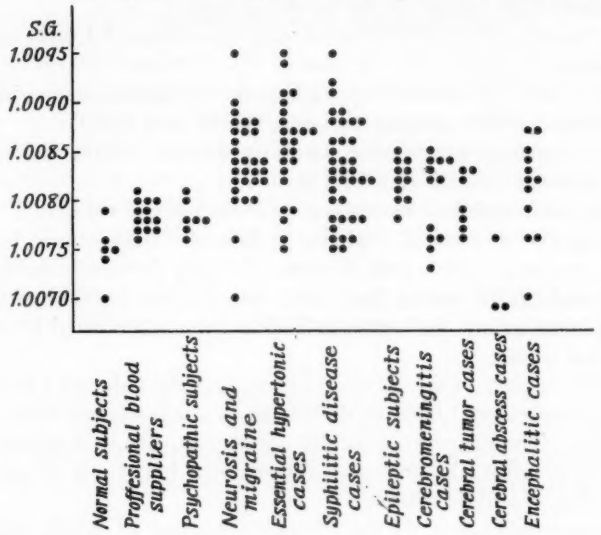
Normal Subjects Cases of diseases in the nervous system were excluded and only subjects with as few complaints as possible were selected. 3 out of the 6 cases tested showed the value of 1.0075 and all the cases had values between 1.0070 and 1.0079, showing a surprising narrow range of fluctuation.

Professional blood suppliers The values in 13 cases ranged between 1.0076 and 1.0081, giving an impression of a slightly supernormal level.

Psychopathic subjects The samples were offered by the Psychiatrics Clinic, Tohoku University, taken from 5 cases of Schizophrenia, 1 of melancholy and 1 of nervousness. The values here ranged also between 1.0076 and 1.0081, tending to rise above the normal value toward the 1.0080 mark.

Neurosis and migraine 17 cases of the 20 neurosis cases had the values ranging between 1.0080 and 1.0088, but one registered the high of 1.0095. The 2 others had values rather lower than the other cases of this group,

Group of the specific gravity in the various diseases



but it is very interesting that the one who showed the low value of 1.0077, a value in the range for the psychopatic group above, was later on diagnosed as a case of Schizophrenia at the Psychiatrics clinic.

The cases of migraine had complaints very closely resembling those of neurosis, such as oppressiveness and vertigo, and in the specific gravity of their liquor showed the values very closely approximating those of the neurotic cases, ranging between 1.0080 and 1.0090.

Essential hypertonic cases Of 24 cases, 19 showed values above 1.0080, 3 values very slightly short of 1.0080 and only 2 normal values.

In particular, the values shown by the cases complaining of vertigo and tinnitus aurium seemed to be distributed in the range higher than 1.0080.

Syphilitic disease cases The values shown by 9 cases of spinal syphilis ranged between 1.0076 and 1.0095, 3 among them registering the " normal values " of 1.0076. These cases showed no increase in the total protein content either. Among the cases not a few showed negative reaction in Wassermann's test, indicating uncertainty in diagnosis, and this may be a reason why the values in this group ranged from the normal to some abnormally high values.

Of the tabes dorsalis cases, all the 8 showed values exceeding 1.0080, the highest registering 1.0088. The easiness of sure diagnosis of this disease

in comparison with that of spinal syphilis seems to have contributed to the uniformly high values shown by this group.

The 2 cases of Erb's paralysis showed the values of 1.0082 and 1.0078 respectively.

The 3 cases of cerebral syphilis and encephalomalacia had values always above 1.0080, ranging between 1.0082 and 1.0092.

The 3 cases of progressive paralysis showed values below 1.0080, namely, between 1.0075 and 1.0078.

A case with syphilitic meningeal neuritis had the value of 1.0080.

Among the 8 cases of syphilis, 5 showed values above 1.0080, the highest registering 1.0091, and the cases showing the nearly normal values of 1.0075 and 1.0078 among the 3 with values below 1.0080 had no subjective complaints at all, their disease having been ascertained by serological examination alone.

Epileptic subjects Only of the 11 cases had the value of 1.0078, all the other cases exceeding 1.0080 in their values, which ranged between 1.0080 and 1.0085. The distribution of the values was thus in a narrower range than in the neurosis, the migraine, the essential hypertony groups and the cases of other syphilitic diseases.

Cerebromeningitis cases Of the 12 cases including 10 of tuberculous meningingitis, 1 of serous meningitis and 1 of another type, the values ranged between 1.0072 and 1.0085. The nearly normal values in this group were registered by the one case of serous meningitis and by the cases complaining of no turbidity of consciousness among the tuberculous meningitis patients.

Cerebral tumor cases One case among the 7 had cerebellar tumor. These cases showed the values ranging between the seemingly normal 1.0076 and the high 1.0083.

Cerebral abscess cases Only 3 cases with their abscess not bursted in the liquor were subjected to the test. One case showed the normal value of 1.0076 and the 2 others the somewhat subnormal value of 1.0069.

Encephalitic cases The values of 10 epidemic encephalitis cases, 1 lethargic encephalitis case and 1 case of another type, 12 in total ranged over the wide range of 1.0070 at the lowest to 1.0087 at the highest.

Various spinal disease-cases Relatively high values were shown by disseminative myelitis and spinal tumor cases and values barely exceeding 1.0080 by the cases of hernia of the intervertebral plates and of amyotrophic lateral sclerosis, but I will refrain from going into details here.

Other disease cases Rather high values were shown by the cases of multiple myeloma and uremia, and in the next place, of Ménières syndromes, of Parkinsonism, of chronic nephritis etc., but the details will be omitted.

The Relation between the Specific Gravity and the Total Protein Content of Spinal Fluid

The total protein content of the spinal fluid was measured by nephelometric method by a Beckman's photoelectric colorimeter. In the cases of professional blood suppliers, psychopathic, neurotic, migranic, hypertensional, lentic, epileptic, cerebrotumorous and cerebroabscessal subjects, where the total protein content is usually below 50 mg/dl and only rarely rises to about 70 mg/dl, we may state in summary, we could not observe any tendency of the values of the specific gravity and the total protein content of the spinal fluid to run in parallelism.

Among the cerebromeningitis and the encephalitis cases, those showing the total protein content of above 100 mg/dl showed the tendency of high specific gravity.

Discussion and Conclusion

The past estimates of the specific gravity of normal spinal fluid have been extremely varied by individual authors, fluctuating in the wide range of 1.001 to 1.010.

Wechsler[3] reported the value to stand between 1.006 and 1.008, Takada[4] as between 1.005 and 1.007, and Dana[5] gives the value of 1.007. Few books contain any full statement on the specific gravity of spinal fluid, except that by Levinson,[6] in which the normal value reported by many predecessors and the same as measured by himself, namely, the value 1.007–1.008 reported by Halliburton, 1.002–1.008 by Kafka, 1.0078 by Zdarck, 1.005–1.007 or 1.012 on the average by Polanyi, 1.0073–1.008 by Nawratski and 1.0064–1.0070 by Levinson as measured with a pycnometer. Of these values, those given by Nawratski and Levinson draw my interest in that they are very small in fluctuation and are approximate to the values I have obtained.

In looking over the results of my tests of more than 200 subjects, although only patients with complaints of diseases of the nervous system or of neurotic symptoms were chosen, the maximum deviation ranged only between 1.0068 and 1.0095. It may be seen, however, upon looking through the values found in the groups of diseased cases as shown in the figure; that there is a clear transition from the normal to intermediate to pathological values, however narrow this range of deviation may be. Namely, from the above results, the normal value may be determied as 1.0070–1.0075, and we may presume that there are no pathological values within this range. The intermediate values are those between 1.0075 and 1.0080, among which normal and pathological cases may be intermingled. The cases showing the values of specific gravity exceeding the line of 1.0080

may be reasonably diagnosed as being pathological cases without exception.

These data may not only serve in diagnosing many diseases, but also in suggesting a new direction to treatment, and moreover, in the surgical field, an improvement in the idea on the specific gravity of the spinal anaesthetics, for the fundamental conception in choosing the appropriate specific gravity of the anaesthetics is based on the assumption that the spinal fluid stands always between 1.006 and 1.008.

For measuring the specific gravity of spinal fluid, which shows only very delicate fluctuation in any case, it is extremely unreasonable to use a floating hydrometer with only 4 equal divisions calibrated between 1.001 and 1.010. It is quite natural that such a means of measurement is quite meaningless in measuring the specific gravity of spinal fluid.

If we are to understand the correlation between the mental and the bodily phenomena through the actions of the autonomous nerve system and the endocrine system, the instability of the bodily reaction in some neurotic and migrainal cases would suggest an anomaly in metabolism as the main factor causing the rise in the specific gravity of spinal fluid. Of course as an organic anomaly would not prevent a rise in the specific gravity, we must pay attention also to the possibility of the existence or a complication of such an anomaly.

The cases of essential hypertony also showed values very closely approaching those of the cases of neurosis and migraine, except a few in which the values were normal or intermediate. All these diseases are common in that they are closely related to hereditary dispositions as well as the ever complicating social environments, but in essential hypertony cases, the chief cause of supernormal specific gravity of the spinal fluid probably lies in the disturbance of the cerebral blood flow, the degeneration of the cerebral blood vessels or the anomaly in the circulatory system.

The cases with syphilitic diseases, epilepsy, cerebral tumors and other chronic diseases, though there are a few exceptions with values distributed in the normal range, usually belong to the group with pathologically high specific gravity of spinal fluid, while in the cases of cerebral meningitis, encephalitis and other acute diseases, the values are scattered over a wide range including the normal and pathological. I presume that this scattering is due to the change in the values by the stadia of the acute inflammatory progress.

It may be that protein and other organic matters and inorganic matters as well as cellular constituents that have influence on the specific gravity undergo delicate changes in quantity due to various reasons related with the complex mechanism of secretion and absorption of spinal fluid, but we may also infer that by the coordinated actions of these various components, the change in the specific gravity is kept in the minimum possible

range. As may be seen from the above experimental results, a light increase of the protein content does not influence the specific gravity. The change in the liquor specific gravity is probably effected in much greater part by the cooperative action of the non-protein ingredients.

From the above results, I am let to believe that I could turn a new page in the research on the specific gravity of spinal fluid and give it a new significance as a means in diagnosing and treating some diseases.

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A Survey on Keratinolytic or Keratinophilic Molds from Soil in Japan

Report I

On *Keratinomyces ajelloi* Isolated from Soil in Japan

By

Miyoko Kominami

(小南 みよ子)

From the Department of Dermatology, Faculty of Medicine, Nihon University. Director: Prof. O. Miura, Tokyo

(Received for publication, January 10, 1957)

As the study of keratinolytic or keratinophilic molds has practically been neglected in Japan, collection and identification of this kind of mold were made. Having isolated three strains of *Keratinomyces ajelloi* which was first discovered by Vanbreuseghem (1952)[1] in Belgium and was isolated later by Ajello (1953)[2] in U.S.A., the writer wishes to write a brief description as her first report in this series.

Experiments

1) Collection of materials and isolation of mold:

Soils were collected from fourteen different parts in the Ueno-Zoo, two dumps in Nihon University Hospital, Tokyo, and dumps in Fujisawa, Kanagawa Prefecture. Following the methods applied by Vanbreuseghem,[1] the above materials were put in sterilized Petri-dishes, half filled and were moistened with sterile distilled water, then human hairs previously washed with soap were placed over this materal and dishes were placed in room temperature. Human hairs put on stable soil were covered with yellowish powdery mold in 3 weeks. On examination the hairs were found to be eaten away or broken, which could be seen with the naked eye. Pure culture was obtained by placing on Sabouraud's glucose agar. We carried out the same experiment with soil from deer and crane stables, and obtained the same results.

2) Research on keratinolytic power:

a) Having washed human hairs in soap and steeped them in a mixture of ether and alcohol for 30 minutes we placed a piece of the hair on a slide in such a manner that the hair did not touch the surface of the slide, and sterilized it by autoclaving at 15 lbs. pressure for 15 minutes, and placed it in Petri-dish containing a sheet of moistened filter paper on the bottom.

On the middle part of the hair a platinumloopful of mold was inoculated, and placed in an incubator at 25°C. In a week's time, it was found that the hair had broken in the attacked portion by digestion, and this was confirmed by microscopic examination.

b) When this mold at 25°C was inoculated on Page's horn agar[3] a transparent zone around the mold began to appear; after three days the diameter of the clear zone was five mm. On the fourth day it was 15 mm., the fifth day 20 mm., the sixth day 23 mm. and the seventh day 25 mm.

3) Macroscopic observations:

a) Placed on Sabouraud's glucose agar at 25°C, this mold could be seen on the second day, on the third day the diameter of the colony was 5 mm., on the fourth day 13 mm., the sixth day 25 mm., the seventh day 33mm. and the fourteenth day 55mm. The surface was powdery or velvety. The color of the colony was first white, but on the third day it began to color and gradually became cinnamon buff[4] from the center to the circumference. On the fourth day the diameter of the colored zone in center was 6 mm. The reverse was white for three days and on the fourth day 3 mm. in the center became victoria lake[4] and was spread gradually into the agar medium.

b) Inoculated on malt extract agar, the result was practically the same as on Sabouraud's agar.

c) On blood agar at 25°C, the color of the colony was lighter than that on Sabouraud's agar. And in two weeks the color of the blood became greenish.

d) On potato agar the result was practically the same as on Sabouraud's glucose agar and on blood agar.

e) On Czapec-Dox agar the growth was slow and the color lighter, but the surrounding agar became brownish in color.

4) Microscopic observation:

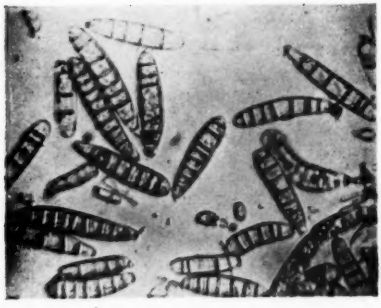
After one week's growth on Sabouraud's glucose agar at 25°C the width of the hyphae was about 1.5 μ and the length of each cell was about 4 to 5 μ. The hyphae branched and at the tip of the branches there were spindle-shaped multicellular macroconidia (Fig. 1). The size of the macroconidia varies from 6 to 11 μ by 20 to 50 μ. The average size was 10 by 46 μ. having as many as 8 or 12 (rarely 6 or 16) cross-walls. The end of the grown macroconidia was slightly rounded (Fig. 2).

5) Pathogenicity test:

Five young guinea pigs were shaved on both sides of the abdomen and numerous short, parallel, superficial incisions were made with the point of a sterile scalpel. A saline suspension of the mold was rubbed into the scarification with the flat side of a scalpel blade. The health of the guinea pigs began to improve and their weight increased. Their

Fig. 1.

Fig. 2.



dandruff was examined but there was no sign of the mold and the recultivation was negative. A week after this treatment a piece of skin was taken from the abdomen of a guinea pig and fixed with formalin, and embedded in paraffin to prepare a histological specimen for Gram, PAS and hematoxilin-eosin staining, and under the microscope there was no sign of mold.

6) Fermentation reaction:

Peptone solution containing B.T.B. as an indicator and 0.5 percent respectively of each of the following carbohydrate substances was provided. The carbohydrate substances are arabinose, xylose, rhamnose, glucose, fructose, galactose, mannose, saccharose, maltose, treharose, lactose, raffinose, dextrine, starch, inulin, glycogen, glycerin, adonitol, dulcitol, mannitol, sorbitol, inositol and salicin. 2 cc. of each mixture was filled into small test tubes in which have previously been placed the inverted Durham's gas tubes, and were sterilized in the steamer at 100°C for fifteen minutes on each of three successive days. Each tube was inoculated with the test mold, and kept at 25°C. Gas production and discoloration of B.T.B. were tested. In the course of 2 weeks no gas was produced in any tube and acid production was practically negative. The result is shown in

Table I. In the table (−) sign means no color change, (+) color change and (±) color change uncertain.

TABLE I

Acid Production

Substratum	Days					
	1	2	3	5	7	14
Xylose	±	±	±	±	±	±
Arabinose	±	±	±	±	−	−
Rhamnose	−	−	−	−	−	−
Glucose	−	±	±	±	±	±
Fructose	±	+	±	±	±	−
Galactose	±	±	±	±	±	±
Mannose	−	−	±	±	±	+
Saccharose	−	−	−	−	−	−
Maltose	±	+	+	+	±	±
Lactose	−	−	−	−	−	−
Trehalose	−	−	−	−	−	−
Raffinose	±	±	±	±	±	±
Dextrin	−	−	−	−	−	−
Starch	−	−	−	−	−	−
Inulin	±	±	±	±	±	±
Glycogen	−	−	−	−	−	−
Glycerin	±	+	+	±	±	−
Adonitol	−	−	−	−	−	−
Dulcitol	−	±	±	−	−	−
Mannitol	±	+	+	±	±	±
Sorbitol	−	+	+	−	−	−
Inositol	−	−	−	−	−	−
Salicin	−	−	−	−	−	−

DISCUSSION

All three strains isolated from the stable soil of Ueno-Zoo, attacked the human hair, but have no effect on guinea pig by skin inoculation, and the appearance of the colony was velvety or powdery though white at the beginning, by the fourth day colony's color began to be cinnamon-buff, and the reverse became victoria-lake, and agar began to be stained. The above is similar to Vanbreuseghem's *Keratinomyces ajelloi*, but the color is slightly different. From the above experiments only spindle-shaped multicellular macroconidia were found. The number of cross-walls were less than those described by Vanbreuseghem, but the size was about

the same. The end of the macroconidia of Vanbreuseghem's strain was pointed but the one above mentioned was rounded. On comparing with the original strain given to Prof. Miura by Prof. Vanbreuseghem, it was practically the same. The effect on the hair was practically the same as described by Prof. Ajello.[2] The writer believes that the three strains are identical with those isolated by Vanbreuseghem.

Summary

1. By means of Dr. Vanbreuseghem's method, three strains of keratinolytic mold were isolated from the stable soils of Ueno-Zoo in Tokyo.

2. These strains were identified as *Keratinomyces ajelloi* Vanbreuseghem.

3. It was proved that the strains have a negative fermentation reaction.

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Suppression of Color Contrast and Retinal Induction by Mechanical Pressure Applied to the Eyeball

By

Tadashi Kohata

(木 幡 正)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, January 11, 1957)

Introduction

Color and brightness contrasts are familiar visual phenomena, but their mechanisms have long remained obscure. Motokawa[1)-4)] found a phenomenon which is very likely to represent a retinal process underlying contrast and other similar visual phenomena, and termed it retinal induction. This phenomenon can be found not only in the human eye, but also in mammalian retinas.[5)] Further it was shown that retinal induction was caused not only by colored light, but also by intermittent white light[6)] and by alternating currents.[7)]

Two kinds of retinal induction were distinguished by Motokawa; the induction effect remaining within a pre-illuminated retinal area after exposure to colored light is called direct induction, and the one set up around the retinal area pre-illuminated is called indirect induction. Direct or indirect induction usually remains unchanged for about 20 seconds, but is extinguished by a second exposure of the retina to a stimulus of complementary color. This phenomenon is called neutralization of retinal induction

Motokawa and Ebe[8)] showed that a pre-established retinal induction could not be abolished by antidromic stimulation of the optic nerve, and Nakagawa and Kohata[9)] found that a pre-established retinal induction could not be abolished by illumination with white light of very high intensity.

In a previous paper[10)11)] we studied the effect of a mechanical pressure applied to the eyeball upon the excitability of the eye and upon the supernormal electrical excitability caused by colored light. The present investigation represents an extension of the preceding one and deals with the effect of mechanical pressure upon retinal induction, and color contrast.

Experimental

Method

After a preliminary dark adaptation for about 20 minutes, experiments were carried out. The method used was essentially the same as that described in the previous papers.[4)10)12)] It consisted in measuring the electrical sensitivity of the dark-adapted eye after a brief illumination, taking the least perceptible electrical phosphene as an index.

Electrical thresholds were determined with the method of limits of ascending order. The stimulating voltage was raised, from a subthreshold value, at first with gross steps and then with smaller steps of about a few per cent until some misty sensation was aroused by the test shock. The final determination of a threshold depended on a comparison procedure in which the effect of a stimulus was compared with that of a control stimulus of zero voltage.

In this way, electrical thresholds of the eye were measured with and without pre-illumination. The effect of illumination was expressed in terms of percentage increases of electrical excitability (reciprocals of threshold) above the resting level. The target used was 10.5 mm. in diameter or 2° in visual angle, and was illuminated from behind by spectral light or white light. The duration of illumination was 2 seconds, if not otherwise stated. The distance from the stimulating surface to the subject's eye was always 30 cm.

The method for applying mechanical pressure to the eyeball was described in detail in the preceding papers[10)11)] so that it seems unnecessary to repeat the same description here. In this apparatus the degree of pressure is expressed in grams. In the present experiment, the greatest pressure used was 200 g. and its duration was 2 seconds. It is to be noted that a pressure of 480 g. just stops the pulsation of the central retinal artery.

Results

1. *Is retinal induction affected by repeated stimulation with strong white light?*

The direct induction caused by a colored light within an illuminated retinal area is revealed by a subsequent white test light, because the curve of electrical excitability for the white test light is modified by the preceding colored light. The strength of induction is measured by the difference between the excitability curve for the pair of colored and white lights and that for the white light alone. Nakagawa and Kohata showed that retinal induction was not affected when a strong white light was interposed between the inducing colored light and the subsequent white test light. The question now arose whether or not a pre-established retinal induction was destroyed by repeated stimulation with strong white light. The inducing

colored light (R) used was red spectral light of 650 mμ, and the white test patch (Wh) was 10 lux and 1 mm. in diameter. The interposed white light (W) was much stronger than any of the inducing and the test lights. The result is shown in Fig. 1, in which a low curve marked by crosses represents the excitability curve for the white test light alone. As is indicated

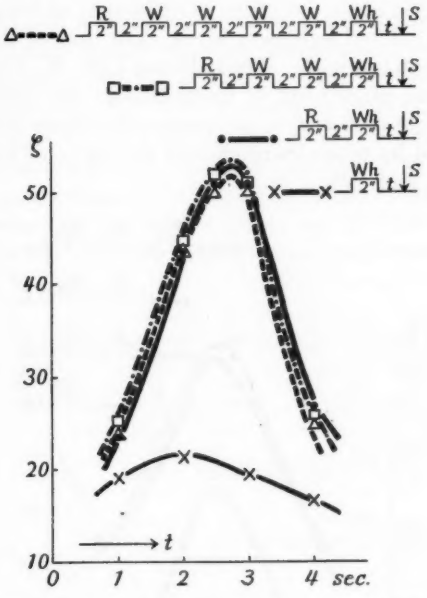

Fig. 1. Experiments showing stability of retinal induction. In the interval between inducing red light (R) and white test light (Wh) 2 to 4 white stimuli (W) were interposed. Ordinates: Percentage increase of electrical excitability (ζ). Abscissae: Time after white test light (Wh). Temporal sequence of stimuli were shown in inset where S and t denote electrical test shock of 0.1 sec. and interval between Wh and respectively. All stimuli were applied to one and the same retinal area.

in the inset, in one experiment two white light stimuli were interposed between R and Wh, but the excitability curve obtained was found little different from the control curve obtained by the pair of R and Wh. In another experiments four white light stimuli were used, but no effect of the interposed white stimuli could be found upon the retinal induction; the latter, once started, can remain unchanged for a considerably long time, however strongly the retina is excited by white light. It is, however, curious that retinal induction is easily extinguished by subsequent

exposure of the retina to the weakest complementary color, as was shown by Motokawa[2].

In the following experiment a colored light of the same quality as the inducing light was interposed between the latter and the white test light in order to see whether or not the retinal induction caused by the inducing light was further strengthened. The result was negative; little change could be found in the curve of induction so long as the intensity of the interposed colored light was lower than that of the inducing light. An example is shown in Fig. 2. The inducing light, R_1, was a strong filtered light (630–670 mμ) of 200 lux in intensity and the interposed light, R_2, was red spectral light (650 mμ) of an intensity five times the foveal threshold. The curve marked by triangles represents the curve of induction obtained by the use of R_2 and Wh.

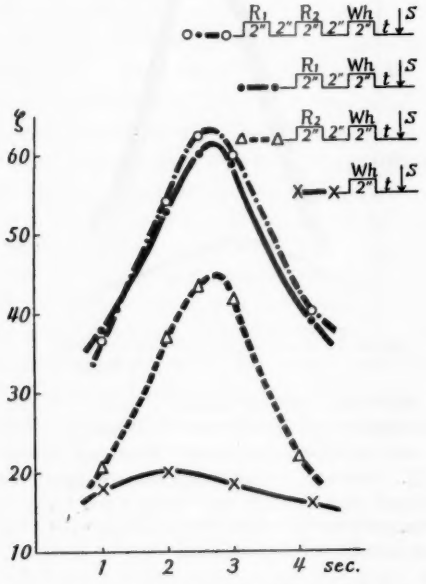

Fig. 2. Further experiments showing stability of retinal induction. A light (R_2) of the same color as the inducing red light (R_1) was interposed between R_1 and Wh. Retinal induction caused by R_1 is little altered by R_2. Other explanation is as in Fig. 1.

The chain curve connecting empty circles represents the curve obtained by the series of stimuli R_1, R_2 and Wh. The continuous curve marked by dots represents the one obtained by the stimulus sequence R_1 and Wh. It is obvious that these two curves coincide with each other

within the limits of experimental error. The experiments mentioned above demonstrate a high stability of retinal induction. In contrast with this, the retinal induction shows a great susceptibility to a circulatory disorder in the retina, as will be reported in the following

2. *Suppression of retinal induction by mechanical pressure applied to the eyeball*

As was described in the previous papers, the electrical excitability of the eye is reduced by mechanical pressure to the eyeball, and no increase in electrical excitability occurs following illumination, when the eyeball is subjected to a pressure from 200 to 250 g. The electrical excitability depressed by a pressure does not recover promptly after removal of pressure, but it takes a time of a few to several seconds for complete recovery, depending upon the intensity and duration of pressure. With the magnitude of pressure used and a duration of 2 seconds, recovery was found complete 8 seconds after removal of pressure. This can be seen also in Fig. 3, in which the continuous curve marked by squares represents the effect of the

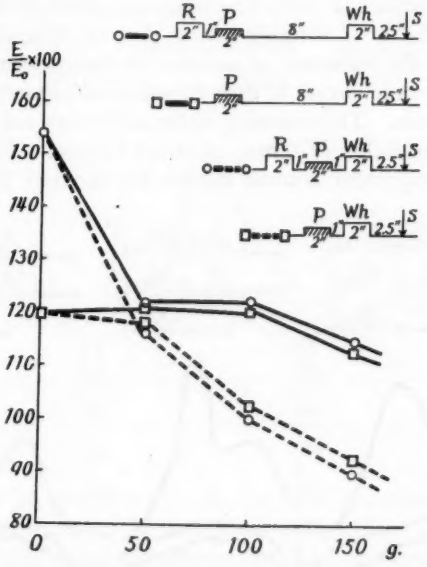

Fig. 3. Experiments showing high susceptibility of retinal induction to mechanical pressure applied to eyball. E : electrical excitability after illumination and application of mechanical pressure. E_0 : Electrical excitability of resting eye. Abscissae : Pressure. Difference between curve marked by circles and that marked by squares represents retinal induction. Temporal sequences of stimuli are shown in inset where R and P represent red inducing light and pressure respectively.

white test light, Wh, preceded by pressure stimuli of various degrees. The ordinate of the curve is expressed in per cent of the electrical excitability without pre-illumination. The white test light brought about supernormal excitability of about 120 per cent irrespective of the magnitudes of pressure. When the inducing light, R, was given 1 second prior to the pressure, the other precedure being the same as above, the supernormality caused by the white test light was found to be the same as before; in other words, no retinal induction appeared, as is shown by the continous curve marked by circles. The supernormality was higher than that for the test light alone when no pressure stimulus was given, that is, retinal induction could be seen without pressure. From these experiments it is obvious that the pressure stimulus following the inducing light destroyed the retinal induction set up by the inducing stimulus. It is to be noted that a pressure as small as 50 g. is effective to depress retinal induction. The results represented by broken curves in Fig. 3 refer to experiments in which the interval between the pressure stimulus and the white test light was only one second. In these cases the effect of the white light was reduced as the intensity of pressure increased. It is without saying that no retinal induction could be noticed under the influence of pressure in these cases.

In Fig. 4 the time curves of direct inductions caused by various spectral lights are shown. The inducing lights used were red (650 mμ), green (530 mμ) and blue lights (470 mμ) of equal energy. The broken curves connecting dots represent control curves for the test light, Wh, alone.

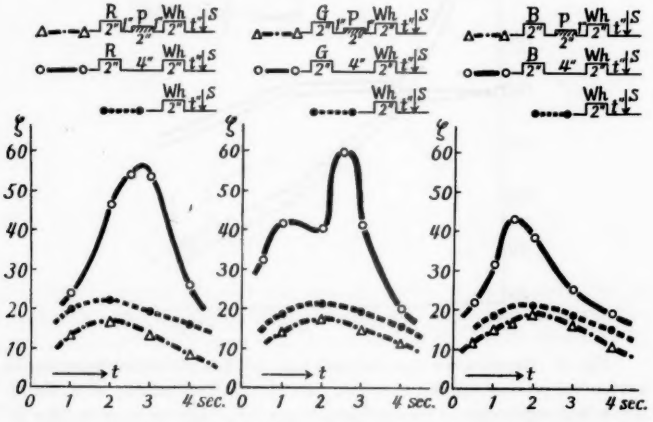

Fig. 4. Time courses of direct retinal induction caused by red (R), green (G) and blue (B) lights. Ordinates: Percentage increases in electrical excitability above resting value measured without any stimuli. P: Pressure of 50 g. R, G and B are spectral lights of equal energy.

The curves connecting open circles represent induction curves obtained by successive stimuli, colored and white. The crest of the curve appears at 2.75 seconds and 1.5 seconds respectively when the inducing light is red and blue. The curve for the green inducing light shows two humps at 1 and 2.5 seconds. In any case the form of the curve of retinal induction conforms to that of the curve which would be obtained with the colored light complementary to the inducing one. All these characteristics of the curves are lost sight of, when a pressure stimulus of 50 g. is interposed between the inducing and the test lights; the curves obtained under this condition are parallel to the control curve for the white test light alone and even lower than this.

The data presented above refer to the direct retinal induction found within the retinal area illuminated by the inducing light. Similar data concerning the indirect induction around the illuminated area are shown in Fig. 5. The white test light was presented at a distance of 3 mm. from

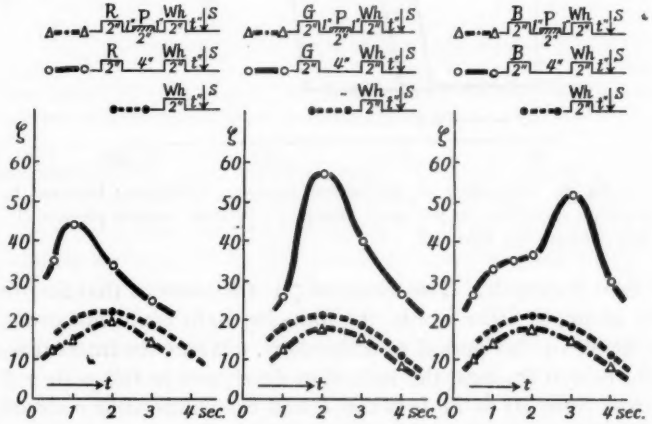

Fig. 5. Time courses of indirect retinal induction. All explanations as in Fig. 4.

the margin of the pre-illuminated area in these experiments. The curve of indirect induction caused by a given inducing light is in shape complementary to that of direct induction caused by the same inducing light. A striking effect of mechanical pressure on indirect induction can be seen in this figure, too (cf. continous curves with chain curves).

In the following experiments after-effects of mechanical pressure upon retinal induction were investigated. The interval between the pressure stimulus and the white test light was so chosen that the effect of the test light was not affected directly by the pressure stimuli; the interval was

at least 6 seconds. The interval between the pressure stimulus and the inducing colored light was varied in order to see how long the depressant after-effect of the pressure upon retinal induction lasted. The result is illustrated in Fig. 6. At an intensity of pressure 50 g., the percentage increase (ζ) of electrical excitability over that of the dark-adapted eye was 20 when the interval between the pressure and the inducing light was

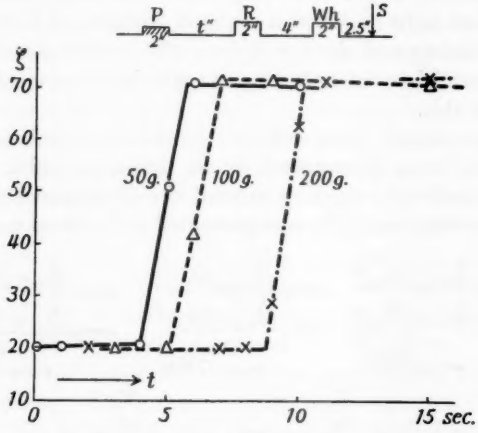

Fig. 6. After-effect of mechanical pressure. Ordinates: Increases in electrical excitability in per cent. Abscissae: Interval between pressure (P) and inducing red light (R).

shorter than 4 seconds. This value of ζ is the same as that for the white test light alone, in other words, the inducing light could not evoke no induction at all in this period of after-effect. When the interval was lengthened to over 6 seconds, the induction developed in full scale: ζ values steeply rose from 20 to 71 between 4 and 6 seconds after removal of the pressure. The depressant after-effect lasted longer, as the intensity of pressure was increased, for example, the after-effect disappeared 7 and 11 seconds after removal of pressure for intensities of 100 and 200 g. respectively.

3. *The effect of mechanical pressure upon color contrast*

Although there is no doubt about the depressing effect of mechanical pressure upon retinal induction, it is a question whether or not the visual phenomenon of color contrast is likewise depressed by the mechanical pressure. Some experiments were done for this question.

Simultaneous color contrast was produced by the well-known method of colored shadows (Goethe, Fechner, Hering and others). The arrangement of this experiment is illustrated schematically in Fig. 7. In front

of a white screen there stand a rod (B) and two white lamps, WL_1 and WL_2. The lamp WL_2 is covered by a red filter R. F. so that the screen is illuminated simultaneously by white and red lights.

On the screen two shadows of the rod, R and G, are seen. The rod casts the shadow R by interrupting the white light, and the G by interrupting the red light.

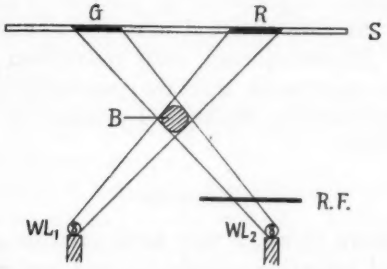

Fig. 7. Arrangements of experiment of colored shadows. S: White screen. R. F.: Red interference filter. B: Rod. WL_1 and WL_2: Tungssten lamps. R: Red shadow. G: Green shadow. Intensity of WL_1 was reduced by a neutral wedge till colored shadows became conspicuous.

The shadow R looks reddish, while G looks bluish-green. The coloration of G is due to simultaneous color contrast. The ratio of the intensities of the white and the red lights has to be adequate to produce conspicuous color contrast by this method. The intensity of white light was controlled by means of a neutral wedge till a conspicuous contrast appeared.

When the subject pressed his eyeball with the tip of a finger till the color shadows disappeared, he could still perceive the rod against the background. Three subjects said that the colored shadows disappeared while the other things were still clearly visible. Then, the question arose as to whether the disappearance of the colored shadows might be due to some disturbance of accomodation brought about by the pressure. Therefore the visual image of each subject was blurred artificially by means of glasses of high refractive power, and asked each subject to compare the visibility of the colored shadows and that of the other things in the visual field. They answered that all the visible things were equally blurred, but that no selective disappearance of the colored shadow could be noticed in this case.

In one of 4 subjects, mechanical pressure could, however, cause no selective disappearance of the colored shadows; he could perceive the colored shadows under pressure so long as he could perceive the other

things in the visual field.

The selective abolition of the colored shadows seemed to depened not only upon the intensity of pressure, but also upon the intensity of contrast. When the intensities of the white and the red lights were decreased in the same proportion, the contrast became less conspicuous, and under such condition the contrast was easier to be abolished by the pressure of the eyeball.

In this psychological experiment the number of subjects was not sufficiently large, and the data obtained were not so quantitative as in the other experiments. In consequence, some reservation should be made to conclude from this experiment that the psychological phenomenon of color contrast is depressed by mechanical pressure in the same degree as is the retinal induction.

DISCUSSION

As has been shown above, a very weak pressure applied to the eyeball abolishes retinal induction completely, and under certain conditions simultaneous color contrast seems to be abolished by the pressure, too.

The retinal induction is so sensitive to pressure that it does not appear even when the effect of light to increase electrical excitability above the resting level is still retained. Fig. 3 shows this situation clearly; under a pressure of 50 g. the supernormal excitability caused by the white test light alone is the same as without pressure, but no retinal induction can be seen under this pressure. According to Motokawa, Iwama and Ebe,[5] the best physiological condition of an animal was required to make experiments on retinal induction. When the condition of the experimental animal was becoming worse owing to unfavorable conditions such as bleeding, deep narcosis, etc., the retinal induction disappeared first, and then the effect of light to increase electrical excitability, the spike-response to a photic stimulus and spontaneous spike discharges disappeared in the order.

Taking advantage of the well-known entoptic phenomenon,[13] the movement of red blood corpuscles in the retinal capillaries can be observed when we look up into the blue sky or when the eye is illuminated by strong parallel rays of blue light.

With the same pressure apparatus used in the present experiment we made some preliminary experiment on the effect of mechanical pressure upon the movement of the red blood corpuscles in the retinal capillaries, and it was found that the rate of flow can be reduced to about 50 per cent by a pressure of 50 to 100 g. This observation justifies the interpretation that the disappearance of retinal induction caused by mechanical pressure is due to anoxia in the retinal tissue.

Summary

The electrical excitability of the dark-adapted eye is increased following an illumination. The effect of a white test light to increase the electrical excitability is remarkably modified when it is preceded by a conditioning colored light. This modification is measured by the difference between the effect of the white light preceded by colored one and that of the white test light alone. When the conditioning and the test stimuli illuminate successively one and the same retinal area, the effect is called direct induction. It is called indirect induction, when adjacent areas are illuminated by the two lights.

1. It was shown that direct induction was little affected when the eye was repeatedly excited by strong white light in the interval between the conditioning and the test lights.

2. Direct or indirect induction was shown to be very sensitive to a mechanical pressure applied to the eyeball. A pressure of 50 g. was found to be sufficient to abolish the induction when it was applied to the eye in the interval between the conditioning and the test lights. A pressure of 480 g. just stopped the pulsation at the central retinal artery.

3. The depressant effect of a mechanical pressure upon retinal induction outlasted the pressure. Complete recovery occurred in 6, 7 and 10.5 seconds after removal of pressure, 50, 100 and 200 g. respectively.

4. Simultaneous color contrast was produced by the method of colored shadows, and its susceptibility to mechanical pressure was investigated. In three of four subjects colored shadows were selectively abolished by a mechanical pressure applied to the eyeball when the intensity of inducing colored light was not too high.

5. The inhibitory effect of mechanical pressure was interpreted as due to anoxia caused by reduction of the rate of blood flow in the retinal capillaries, as was evidenced by entoptic observations of the retinal blood flow.

I am greatly indebted to Prof. K. Motokawa for his kind guidance and for many valuable discussions.

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Biochemical Studies on Carbohydrates

CCV. Mucopolysaccharides and Mucoproteins in Pig Lung First Paper: Glucidamins and a Molisch-positive Mucopolysaccharide

By

Katuhiko Tokita
(鴇田 克彦)

From the Medico-chemical Institute (Director: Prof. H. Masamune) and Prof. T. Nakamura's Clinic of Internal Medicine, Tohoku University, Sendai

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In 1949, Utusi[1] separated from pig lungs two glucidamins, of which one was glacial acetic acid-soluble and group active and the other was glacial acetic acid-insoluble and hemopoietic, and heparin. Reinvestigation of hexosamine-containing substances in this organ has been undertaken to carry out on recent principles of tissue fractionation and by the aid of modern methods of analysis. The experiments, which are dealt with in this account, disclosed the presence of three glucidamins, one weakly group A and O active and two group inactive, and a group inactive mucopolysaccharide resembling that isolated by Masamune *et al.*[2] from human lungs.

Experiments and Comments

Material

Fresh pig lungs were freed from adhering adipose tissue and membranes, trachea, bronchi and blood vessels and masticated. 1 kg. of the brei was shaken with 3 *l.* of 10% NaCl for 24 hours under cooling on chopped ice. The red, somewhat viscous, opaque extract was separated after centrifugation and dialyzed in cellophane bags against running water for 2 days, and some precipitate during the dialysis which gave no distinct Molisch reaction was filtered off and discarded. The red-brown translucent filtrate was distilled in vacuo to a thick solution (less than 100 cc.) and precipitated with abs. ethanol, and after washing with 500 cc. of abs. ethanol, placed in a desiccator ($CaCl_2$). Yield 52 g. The tissue residue above was submitted to two more extractions, and the second and third extracts gave 18 and 10 g. substances respectively after similar treatment. Extraction was repeated on two new batches of pig lung brei, one amounting to 1.3 and the other to 1.25 kg., and 300 g. of a dry red-brown material was obtained in total. Molisch ++, biuret ++, Goldschmiedt ±, test for P ±.

Fractionation Procedure

1) Fractionation by means of sodium sulfate. The whole dry material was taken up in 1 *l.* of water, adjusted to pH 7.0 with 10% NaOH, and after standing overnight at room temperature, homogenized, followed by centrifugation. The brown turbid supernatant was set aside, and the centrifugate was further exhausted with 1 and 0.7 *l.* portions of water. The solutions were united, added to with some Celite and filtered under suction. The yellowish and almost clear filtrate was concentrated to about 500 cc. Then it was warmed in a water-bath to 35°C, saturated with 250 g. of anhydrous sodium sulfate, and after adjustment to pH 7.0, stood in an air-thermostat at 35°C for 1 hour. The flocculent precipitate was filtered off with suction in the thermostat. It was again precipitated from 200 cc. of water at the same temperature by saturation with sodium sulfate and filtered off in a similar manner. The filtrates were diluted with double the volume, dialyzed for 3 days, concentrated to 200 cc. and again dialyzed for 2 days to eliminate all the sulfate ions. It was further condensed to a thick syrup (about 1 cc.), precipitated with acetone and washed with acetone and ether. When dried in vacuo over $CaCl_2$, it amounted to 0.75 g.—Fr. I (a yellow powder). The deposit at the sodium sulfate concentration of 33% was dissolved in 300 cc. of water, placed in the thermostat regulated to 25°C and after slowly saturating with 75 g. of the same salt, stood for 1 hour in that thermostat. The deposit here was filtered off with suction and washed with 200 cc. of the satd. sodium sulfate at 25°C, manipulating in the thermostat throughout. The filtrate and washing were diluted doubly and subjected to dialysis and condensation twice successively with subsequent precipitation, washing and drying similar to above. 2.1 g. of a non-sticky, brownish powder was given—Fr. II.

The deposit at the sodium sulfate concentration of 20% was dissolved in 300 cc. of water, dialyzed for 3 days, condensed to 100 cc., dialyzed for 2 days and condensed to a syrup in turn. It was precipitated, washed and dried as above. 0.6 g. of a red-brown powder was obtained—Fr. III.

Chemical tests of Frs. I–III resulted as follows.

Fr.	Molisch	Goldschmiedt	Biuret	Test for P
I	++	±	++	+
II	++	−	++	−
III	±	−	+++	−

(− negative, ± faintly positive, + slightly positive, ++ markedly positive, +++ strongly positive.)

And the assay for aniti-hemagglutinative potency (a glass plate method[8]) revealed that Fr. II was group A active at a dilution of 1:1600 and group O active against an anti-OS saliva serum at a dilution of 1:400, whereas Fr. III which was only faintly Molisch positive was even less group active, suggesting contamination with Fr. II, and Fr. I was group inactive.

From Fr. I, which was soluble in the satd. sodium sulfate at 35°C, was isolated a mucopolysaccharide as will be described below. The remaining markedly Molisch-positive fraction Fr. II was expected to contain a glucidamin or gluci-

damins. It was subdivided as follows, taking blood group activity as an indication.

2) *Fractionation of Fr. II with ethanol of its solution in acetate buffer.* 2.1 g. of it was dissolved in 50 cc. of acetate buffer pH 4.6, I 0.4. The slightly viscous, brown solution was chilled on ice, and 25 cc. of abs. ethanol was added in drops under agitation. The mixture was placed in a refrigerator (0°C) for 2 hours and centrifuged in a refrigerated centrifuge. The yellow, clear supernatant was set aside. The centrifugate was again precipitated from 25 cc. of the buffer with half the volume of abs. ethanol in a similar manner, and after washing with abs. ethanol and acetone, dried in a desiccator ($CaCl_2$)—Fr. II_a. The supernatant fluid here was united with the one above and further fractioned with increasing volumes of ethanol. Yields and results of assays:

Fr.	Pptd. at conc. range of ethanol (% (v/v)*)	Yield (g.)	Group A active dilution	Molisch test	Biuret test
II_a	0–33	1.05	$1:10^2$	+	##
II_b	33–54	0.69	$1:1.6\times10^3$	++	++
II_c	54–60	0.10	$1:\leqq10^2$	++	++
II_d	60–80	0.23	$1:<10^2$	++	++

* It is assumed that the volumes of the buffer-ethanol mixtures were equal to the sums of the volumes of the buffer and ethanol mixed together.

The substance recovered from the mother fluid of Fr. II_d was Molisch negative. The slightly positive Molisch reaction of Fr. II_a was probably due to contaminating Glucidamin I (See below) which was mostly recovered from Fr. II_b. When subjected to electrophoresis in an acetate buffer of pH 4.6, II_b–II_d showed two boundaries, stationary and moving. The areas of the boundaries in the patterns revealed that II_b and II_d are occupied mostly by a moving component and II_c mostly by a stationary component, and moreover, the moving component of II_b migrated faster than the corresponding component of Fr. II_d.

In short, the findings indicated that the main component of II_b was a group active glucidamin and those of II_c and of II_d were a group inactive glucidamin each.

3) *Isolation of the glucidamins and mucopolysaccharide.* a) Glucidamin I. 680 mg. of Fr. II_b was taken up in Michaelis' ammonium chloride-ammonia buffer pH 8.0, I 0.1, and after rejection of a small insoluble part, fractioned with ethanol at 0°C as above. The yields, group activity and Molisch reaction of the divisions were the following.

No. of division	Pptd. at conc. range of ethanol (%)	Yield (g.)	Group A active dilution	Molisch test
1	0–30	Trace		±
2	30–50	0.25	$1:<2\times10^2$	+
3	50–60	0.25	$1:1.6\times10^3$	++
4	60–70	0.10	$1:8\times10^2$	++
5	70–80	0.04	$1:4\times10^2$	±

Div. No. 3, which contained most of the group active glucidamin, gave in acetate buffer pH 4.6 an electrophoretic pattern with one stationary and one moving boundary, suggesting a mixture of two kinds of protein. Therefore, it was subdivided at 0°C with ethanol in acetate buffer pH 6.2 (I 0.4). Subdivisions:

No of subdivision	Pptd. at conc. range of ethanol (%)	Yield (mg.)	Group A active dilution	Molisch test
1	0–40	Trace		
2	40–55	131	$1:1.6\times10^3$	++
3	55–60	48	$1:\geqq2\times10^2$	++
4	60–70	28	$1:<2\times10^2$	+

The most group A active subdivision No. 2 proved to be electrophoretically homogeneous, as shown in Fig. 1. It will be referred to as Glucidamin I.

b) Glucidamin II. 92 mg. of Fr. II_c above was dissolved in 50 cc. of a 5% barium acetate solution in 0.5 *N* acetic acid and chilled to 0°C on ice, 61 cc. of abs. ethanol was added, and the mixture was stood in a refrigerator (0°C) overnight. A trace of the precipitate was centrifuged off in a refrigerated centrifuge and discarded. The supernatant fluid (ethanol concentration 55%) was added to with additional ethanol to 60%, stood overnight at 0°C and centrifuged at 0°C as above. The centrifugate was washed with abs. ethanol twice and with acetone once and dried. It weighed 85 mg. when dry (The mother fluid was free from Molisch-positive substances). This was dissolved in 20 cc. of water, passed through a column (0.8×20 cm.) of Amberlite IR 120 (H^+ form) to remove barium and washed with 50 cc. of distilled water. The effluent (pH 4.8) and washing were distilled in vacuo at below 30°C to syrupy consistency (pH 6.2), precipitated with 100 cc. of abs. ethanol and a few drops of abs. ethanol saturated with anhydrous sodium acetate, and after washing with abs. ethanol and acetone, dried. Yield 70 mg.—Glucidamin II.

c) Glucidamin III. 225 mg. of Fr. II_d was purified in a like manner as Fr. II_c. It was taken up in 40 cc. of the barium acetate solution in 0.5 *N* acetic acid above. The insoluble part was rejected, and the clear solution was chilled and added to with 60 cc. of abs. ethanol whereby slight turbidity occurred. After standing for 5 hours in the refrigerator (0°C), the mixture was centrifuged at 0°C and the supernatant was added to with abs. ethanol to 80%, followed by standing overnight at 0°C. The flocculent precipitate was centrifuged off (The mother fluid showed no Molisch reaction), washed with two changes of ethanol and with acetone in succession and dried. 165 mg. of a yellowish powder given was taken up in 20 cc. of water, the small insoluble part was removed and the solution was treated with Amberlite IR 120 (H^+ form), concentrated, precipitated, washed with ethanol and acetone and dried as in b)—Glucidamin III. Yield 145 mg.

d) The mucopolysaccharide. 725 mg. of Fr. I was taken up in 40 cc. of a 5% calcium acetate solution in 0.5 *N* acetic acid, the yellow-colored insoluble part was removed after centrifugation, and the supernatant was chilled to 0°C,

added to with 10 cc. of abs. ethanol to 20% and centrifuged at 0°C. The centrifugate which was Goldschmiedt positive was rejected. The supernatant was further fractioned by increasing ethanol concentration to 50% and then to 75% in a similar manner. The deposits were washed with abs. ethanol and acetone and dried—Ppt. 1 and 2. Their yields were 260 and 180 mg. respectively. The final centrifuged supernatant fluid was Molisch negative.

When agitated with 90% phenol, Ppt. 1 dissolved mostly. The soluble part recovered showed only faint Molisch reaction. The part undissolved (70 mg.) was slightly Molisch- and markedly biuret-positive and contained phosphorus, suggesting a mixture of nucleic acids and non-glucidamin proteins.

Ppt. 2, which was Molisch +, biuret + and free from phosphorus, was dissolved in 20 cc. of the calcium acetate solution in dilute acetic acid above and chilled to 0°C, 20 cc. of abs. ethanol was added, and the small deposit was discarded after centrifugation in the refrigerated centrifuge. The supernatant at 0°C was precipitated by addition of additional ethanol to 75% and centrifuged at 0°. The centrifugate was washed with abs. ethanol and acetone and dried. It still showed positive biuret reaction, though weak, and was colored deep yellow so that it was dissolved in 50 cc. of water and passed through columns (0.8×20 cm.) of Amberlite IR 120 (H^+ form) and of Amberlite IRA 410 ($CO_3^{=}$ form) in succession (The substance became biuret negative and colorless when passed through the column of the latter resin). The effluent and washing were concentrated, precipitated, washed and dried in the manner described in b). 60 mg. of a hygroscopic, white powder was given. It gave strong Molisch reaction but no biuret reaction.

Properties of the Glucidamins and Mucopolysaccharide

Electrophoresis. All the preparations showed one symmetrical or nearly symmetrical boundary after electrophoresis in acidic and basic buffers, as shown in Fig. 1.

Solubility. a) All of Glucidamins I–III are soluble in water of pH values of 1.6–9.0 and 90% phenol and insoluble in the satd. ammonium sulfate, the satd. sodium sulfate at 33°C, ethanol, acetone and ether, whereas Glucidamin II and III are insoluble in glacial acetic acid contrary to Glucidamin I which is soluble in the same solvent. The glucidamins are not precipitated from water by addition of barium-acetate and -hydroxide. Lead acetate partially precipitates all of them and cupric chloride Glucidamin I only.

b) The mucopolysaccharide dissolves in plain, alkaline an dacidic water, the satd. ammonium sulfate and the satd. sodium sulfate at 33°C, but insoluble in ethanol, acetone, ether, 90% phenol and glacial acetic acid. It is precipitated from water by neither barium-acetate and -hydroxide nor lead acetate nor cupric chloride.

Ultraviolet absorption. The preparations absorbed ultraviolet light each with a maximum at the wave-lengths of 270–280 mμ, which is characteristic to cyclic amino acids, but the strength of absorption differed from preparation to pre-

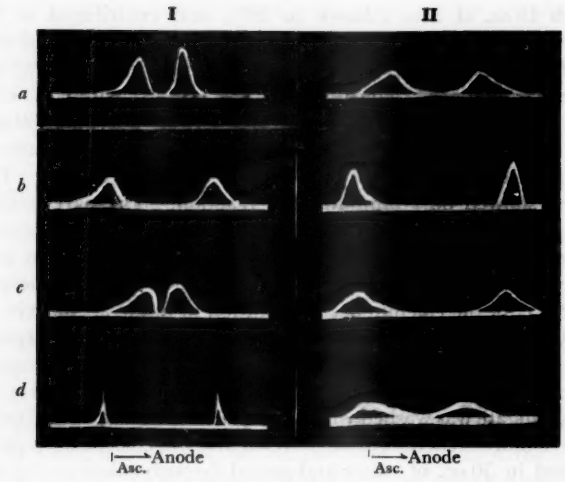

Fig. 1. Electrophoretic patterns (2/3×) of 1% solutions of Glucidamin I (*a*), II (*b*) and III (*c*) and 0.6% solutions of the mucopolysaccharide (*d*) in phosphate buffer pH 7.7, I 0.2 (**I**) and acetate buffer pH 4.6, I 0.2 (**II**). Temperature 15°C; current 8 mA; exposure 75 (**I***a*, **II***a*, **I***b*, **II***b*, **I***c*, **II***d*), 60 (**II***c*) or 45 (**I***d*) minutes after starting current. Mobility:

I*a*	Glucid-amin I	at pH 7.7	$\frac{(-1.20)\times 0.007081\times 0.3}{4500\times 0.008}=-7.1$	$cm^2\cdot volt^{-1}\cdot sec^{-1}\times 10^{-5}$
II*a*		at pH 4.6	$\frac{(-0.65)\times 0.005692\times 0.3}{4500\times 0.008}=-3.1$	"
I*b*	Gl. II	at pH 7.7	$\frac{(-0.75)\times 0.007081\times 0.3}{4500\times 0.008}=-4.4$	"
II*b*		at pH 4.6	$\frac{(0.0)\times 0.005692\times 0.3}{4500\times 0.008}=0.0$	"
I*c*	Gl. III	at pH 7.7	$\frac{(-1.40)\times 0.007081\times 0.3}{4500\times 0.008}=-8.3$	"
II*c*		at pH 4.6	$\frac{(-0.45)\times 0.005692\times 0.3}{3600\times 0.008}=-2.7$	"
I*d*	Mucopoly-saccharide	at pH 7.7	$\frac{(-0.65)\times 0.007081\times 0.3}{2700\times 0.008}=-6.4$	"
II*d*		at pH 4.6	$\frac{(-0.60)\times 0.005692\times 0.3}{4500\times 0.008}=-2.8$	"

Optical rotation in water.

Glucidamin I: $[\alpha]_D^{25}=\frac{-0.408\times 1}{0.0105\times 1}=-38.9°$ (pH 6.6)

II: $[\alpha]_D^{25}=\frac{-0.370\times 1}{0.0100\times 1}=-37.0°$ (pH 6.6)

III: $[\alpha]_D^{25}=\frac{-0.320\times 1}{0.0103\times 1}=-31.8°$ (pH 6.6)

Mucopolysaccharide : $[\alpha]_D^{17}=\frac{-0.258\times 1}{0.0099\times 1}=-26.8°$ (pH 7.2)

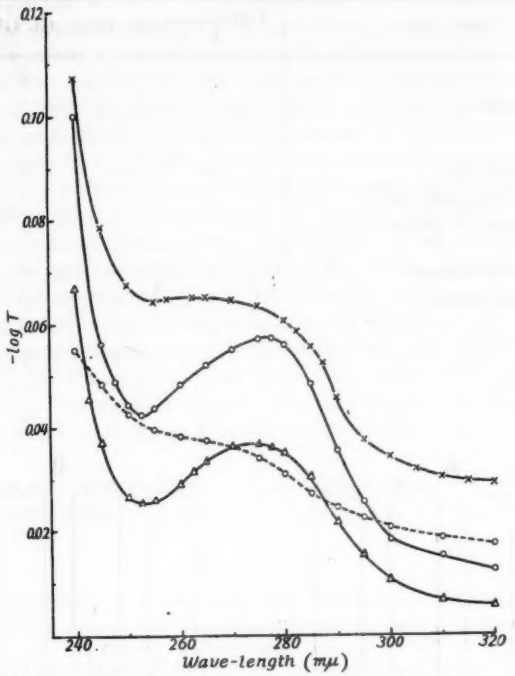

Fig. 2. Ultraviolet absorption of 10 mg% solutions in water of Glucidamin I (—○—○—), II (—△—△—) and III (—×—×—) and the mucopolysaccharide (---○---○---). Cell 1 cm. thick.

paration, as shown in Fig. 2.

Test-tube tests. The results of tests on 0.3% solutions of the preparations are embodied in Table I.

Paper partition chromatography of the components. a) Sugars. 5 mg. of substance was sealed with 0.5 cc. of 1 *N* H_2SO_4 in a tube and heated at 100°C for 5 hours. When cold, the hydrolysate was neutralized with the satd. baryta to pH 5.8. The centrifuged supernatant and washing were distilled in vacuo to dryness and dissolved in 0.05 cc. of water, and 0.02 cc. (0.01 cc. in the case of the mucopolysaccharide) of the solution was applied to chromatography according to Masamune and Yosizawa.[4] As shown in Fig. 3, spots were given at the positions corresponding to galactosamine, glucosamine, galactose, mannose and L-fucose for all the glucidamins, and spots at the positions corresponding to glucose and xylose or lyxose in addition to those corresponding to the sugars above for the mucopolysaccharide.

TABLE I

Test-tube Assay of the Glucidamins and Mucopolysaccharide

Test	Glucidamin I	Gl. II	Gl. III	Mucopolysaccharide
Molisch	++	++	++	++
Osaki-Turumi				
Direct	+	±	+	±
Indirect	−	−	−	±
Neuberg-Saneyoshi	−	−	−	−
Test for hydrolyzable sulfur	−	−	−	−
Bial-Mejbaum for pentose and galactose	±	+	+	±
Dische for deoxyribose	−	−	−	−
Test for phosphorus	−	−	−	−
Biuret	++	++	++	−
Millon	+	+	+	−
Hopkins-Cole	−	±	+	−
Sakaguchi	+	+	++	−

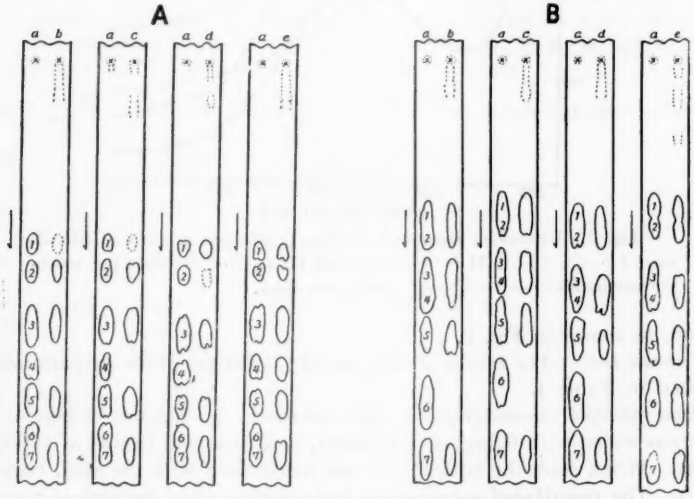

Fig. 3. Sugar paper chromatograms (1/7×) of Glucidamin I (*b*), II (*c*) and III (*d*) and the mucopolysaccharide (*e*). Irrigation effected with Solvent butanol-pyridine-water (5:3:2 by volume) (**A**) or Solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume) (**B**). Temperature of test 22–23°C. Indicator aniline hydrogen phthalate reagent of Partridge. Reference runs (*a*) carried out with 0.02 cc. of a standard solution which was 1% with respect to individual non-amino sugars and aminosugar sulfates.

1 galactosamine ; *2* glucosamine ; *3* galactose ; *4* glucose ; *5* mannose ; *6* xylose ; *7* L-fucose.

b) Amino acids. 5 mg. of substance was hydrolyzed with 0.5 cc. of 6 *N* HCl by heating at 100°C for 24 hours in a sealed tube. The hydrolysate was repeatedly evaporated in vacuo over solid caustic soda to expel the hydrochloric acid as much as possible, adding water from time to time, and finally dried up. The residue was taken up in 0.05 cc. of water (pH of the solution 5.6), and 0.01 cc. (0.02 cc. in the case of the mucopolysaccharide) was sampled on a 30× 30 cm. square of Toyo Roshi filter paper No. 2. Primary irrigation was effected with a mixture of butanol, acetic acid and water in the volume proportions of 4:1:2 and the secondary with 80% phenol in an ammonia-containing atmosphere or with Solvent 0.1 *M* borax-phenol (14:86 by volume) of Masamune and Hakomori[5]. Indicated with 0.1% ninhydrin in butanol satd. with water or 0.1% ninhydrin in an ethanol-glacial acetic acid mixture in the volume proportion of 7:3[5]. All of the glucidamins were revealed to contain aspartic and glutamic acids, glycine, serine, threonine, alanine, tyrosine, valine and/or methionine, leucine and/or isoleucine, proline, phenylalanine, histidine, lysine and arginine. To add, it is to be mentioned that tryptophan has been also detected in Glucidamin II and III by Hopkins-Cole reaction. The hydrolysate of the mucopolysaccharide gave a less number of spots of amino acids: aspartic and glutamic acids, glycine, serine, threonine, alanine, leucine and/or isoleucine, proline and arginine.

Fig. 4 shows the chromatograms of the hydrolysates of Glucidamin I and of the mucopolysaccharide.

TABLE II

Composition of the Glucidamins and Mucopolysaccharide

Analysis	In per cent				In equivalents per equivalent weight			
	Glucidamin I	Gl. II	Gl. III	Mucopolysaccharide	Gl. I	Gl. II	Gl. III	Mucopolysaccharide
Nitrogen*	12.4	11.0	11.2	5.0	16.4	11.1	9.8	3.1
Hexosamine†	9.7	12.7	14.6	20.6	1.0	1.0	1.0	1.0
Galactose‡	7.6	5.8	10.3	8.6	0.8	0.5	0.7	0.4
Mannose‡	4.0	11.6	3.8	8.3	0.4	0.9	0.3	0.4
Glucose‡				2.6				0.1
L-Fucose§	2.4	2.4	1.8	1.8	0.3	0.2	0.1	0.1
Xylose and/or lyxose as xylose//				5.4				0.3
Sialic acid¶	4.0	2.7	4.1	3.7	0.2	0.1	0.2	0.1
Ash**	1.5	0.8	1.9	13.1				

* Micro Kjeldahl. † Masamune and Yosizawa.[6] ‡ Masamune and Sakamoto.[7]
§ A modification of Dische and Shettles method.[8] // Masamune and Sakamoto.[9]
¶ Werner and Odin (Direct Ehrlich)[10]; Calcd. as $C_{11}H_{19}NO_{10}$ (325) (Cf. Blix *et al.*[11]). ** Pregl method without use of H_2SO_4.

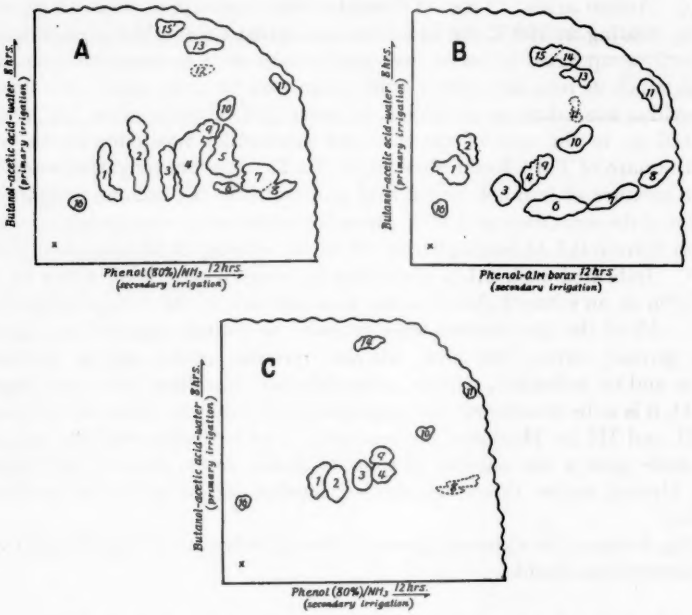

Fig. 4. Amino acid paper chromatograms (1/7×) of Glucidamin I (**A**, **B**) and of the mucopolysaccharide (**C**). Paper squares Toyo Roshi filter paper No. 2. Primary irrigation effected with Solvent butanol-glacial acetic acid-water and the secondary with 80% phenol in an ammonia-containing atmosphere in **A** and **C** or with Solvent 0.1 *M* borax-phenol in **B**. Temperature of test 23°±1°C. Indicated with 0.1% ninhydrin in butanol satd. with water in **A** and **C** and with 0.1% ninhydrin in an ethanol-glacial acetic acid mixture (7:3 by volume) in **B**.

1 aspartic acid ; *2* glutamic acid ; *3* serine ; *4* glycine ; *5* hexosamine ; *6* histidine ; *7* lysine ; *8* arginine ; *9* threonine ; *10* alanine ; *11* proline ; *12* tyrosine ; *13* valine and/or methionine ; *14* leucine and/or isoleucine ; *15* phenylalanine ; *15'* leucine, isoleucine and/or phenylalanine ; *16* a peptide.

Quantitative analyses. The figures are shown in Table II.

Blood group activity. It was assayed on a glass plate according to Masamune *et al.*[3] The results indicated that Glucidamin I only is group active (Table III).

Taken altogether, the three glucidamins are made up of the same components, tryptophan which is absent in Glucidamin I excepted, but differ from one another in analytical figures, electrophoretic mobility and ultraviolet absorption and are not all the same in solubility, precipitability, optical rotation and blood group activity. Judging from their solubility and group activity, Glucidamin I in this account looks to be identical with the glacial acetic acid-soluble glucidamin

TABLE III

Anti-hemagglutinative Potency of the Glucidamins and Mucopolysaccharide

Goat: a normal anti-O goat serum. Anti-OS: an anti-OS saliva serum.

Substance	Red cells	anti-serum	Dilution of the substances (1:)									
			10^2	2×10^2	$2^2\times 10^2$	$2^3\times 10^2$	$2^4\times 10^2$	$2^5\times 10^2$	$2^6\times 10^2$	$2^7\times 10^2$	$2^8\times 10^2$	$2^9\times 10^2$
Glucidamin I	A	α	−	−	−	−	−	+	+	+	++	++
	B	β	±	+	+	++	++	++	++	###	###	###
	O	Goat	+	++	++	++	###	###	###	###	###	###
	O	Anti-OS	−	−	−	+	+	+	++	++	###	###
Gl. II	A	α	±	±	+	++	###	###	###	###	###	###
	B	β	++	++	++	###	###	###	###	###	###	###
	O	Goat	++	###	###	###	###	###	###	###	###	###
	O	Anti-OS	++	++	###	###	###	###	###	###	###	###
Gl. III	A	α	++	++	++	###	###	###	###	###	###	###
	B	β	++	++	++	###	###	###	###	###	###	###
	O	Goat	###	###	###	###	###	###	###	###	###	###
	O	Anti-OS	++	++	++	###	###	###	###	###	###	###
Mucopoly-saccharide	A	α	++	++	++	###	###	###	###	###	###	###
	B	β	++	++	++	++	###	###	###	###	###	###
	O	Goat	++	###	###	###	###	###	###	###	###	###
	O	Anti-OS	++	++	###	###	###	###	###	###	###	###

of Utusi[1], and the hemopoietic glucidamin of Utusi[1] to have been a mixture of Glucidamin II and III.

SUMMARY

1. Three glucidamins and one mucopolysaccharide were isolated from fresh pig lungs in electrophoretically homogeneous or almost homogeneous state.

2. The glucidamins are composed of galactosamine, glucosamine, galactose, mannose, L-fucose, sialic acid and 14 or more amino acids, and the mucopolysaccharide of galactosamine, glucosamine, galactose, glucose, mannose, L-fucose, xylose and/or lyxose, sialic acid, aspartic and glutamic acids, glycine, serine, threonine, alanine, leucine and/or isoleucine, proline and arginine.

3. Such physical properties as solubility, precipitability, ultraviolet absorptivity, optical rotatory power and electrophoretic mobility and blood group activity of the preparations were also examined.

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4) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
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6) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.
7) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
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Chemistry and Biology of Lipids

XXVI. Fractionation of Group Lipid Mixtures of Pig Stomach and Pig Lung by Precipitin Reaction

By

Haruo Numabe

(沼部 治夫)

From the Medico-chemical Institute, Tohoku University, Sendai; Director: Prof. H. Masamune

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Kabat[1] does not believe the presence of group lipids, claiming that the preparations till now are all mixtures of lipid and mucopolysaccharide. However, the reasons for his view is not persuasive, for instance, the weakness of their group activity can not negate that group lipid preparations are single chemical entities, because even among the group mucopolysaccharides some are very weak. In this Institute there have been separated various group lipids from various tissue sources in electrophoretically homogeneous state[2)-10)], and it was revealed regarding them that 1: The "partial group lipid" the group active fraction of human liver group lipid (Group A) contains non-N-acetylated galactosamine[11)] contrary to group mucopolysaccharides in which hexosamine molecules are all N-acetylated, 2: The "partial group lipid" of group lipids from human liver (Group A) and pig lung (Group BO)[12)] were found to be devoid of L-fucose and sialic acid as sugar components notwithstanding that group mucopolysaccharides generally contain them, and 3: The amino group of one ethanolamine residue could not be dinitrophenylated, when the "partial group lipid" of human liver group lipid was treated as such with dinitrofluorobenzene. Moreover, the mucopolysaccharide of pig lung, wherefrom a Group BO lipid is isolable, has been demonstrated to be group inactive.[12a)]

Fractionation of group lipid mixtures which is dealt with in this account was carried out for affirmation of the existence of group lipids in nature. Group lipid mixtures were prepared from pig stomachs and pig lungs and fractioned by precipitation with animal immune sera, which had been prepared by injection of human erythrocytes and human saliva and freed from antibodies having no connection with group substances. The precipitates were divided into the antigen and antibody, and the antigens given were examined physically, chemically and serologically with results perfectly in accord with anticipations.

Experiments and Comments

Preparation of Group Lipid Mixtures

1) Pig stomach group lipid mixture (*Lipid AO*). The mucosa teared off 11 pig stomachs (1850 g. in total) was washed with water, minced and dehydrated by covering under 9 *l.* of 91% denatured alcohol containing 3% methanol for 10 days. It was then dried in a Faust apparatus at 40°C. The dry material thus given (355 g.) was powdered in an iron mortar, boiled with 2 *l.* of 95% alcohol and filtered hot with suction. The tissue residue was further exhausted with 1 *l.* of 95% alcohol in a similar manner. The solutions united were stood in a refrigerator (0–3°C) overnight and filtered again to eliminate the occurring sticky yellowish precipitate. The filtrate here was concentrated in vacuo (37°C) to a syrup, 300 cc. of purified acetone was added under agitation and the mixture was stood in the refrigerator overnight. The acetone-insoluble part was centrifuged off, washed with 30 cc. portions of dry acetone until no colored substances came out and placed in a desiccator (H_2SO_4). 34 g. of an amorphous yellowish hygroscopic powder was given. It was washed with five 30 cc. portions of a mixture of dry ether and dry alcohol in the volume proportion of 2:1. The washings were distilled together to dryness, washed with acetone and dried. It (a brownish powder, 1.4 g.) was Group A active at a dilution of 1:500. The part undissolved in the ether-alcohol mixture was also desiccated. The fine yellowish powder given here weighed 1.9 g. and was Group A active at a dilution of $1:10^5$. This was boiled with 20 cc. portions of benzene under reflux for 5 minutes and filtered hot under suction, and the residue on the funnel was treated in a similar manner three times more. The filtrates were combined, concentrated and desiccated as above to give 0.8 g. of a brownish, gelatin-like substance. It was extracted four times employing 40 cc. of abs. alcohol as was with 20 cc. of benzene, each extraction taking 10 minutes. The solutions filtered hot were stood together in the refrigerator overnight and the deposit was removed by filtration. The filtrate was distilled in vacuo to 10 cc., and 20 cc. of dry ether was added, whereby a precipitate appeared. After standing in the refrigerator overnight, the precipitate was filtered off and dried. 0.3 g. of a light yellow, non-sticky powder was obtained. It was stirred up in 10 cc. of water, and after addition of 0.1 *N* NaOH to pH 8.0, centrifuged. The centrifugate was subjected to two more extractions, each extraction being followed by centrifugation. The supernatant fluids were united and neutralized with 10% HCl, 5 cc. of a 10% solution of $Ba(AcO)_2 \cdot H_2O$ was added and the mixture was stood in the refrigerator overnight. After separating off the occurring precipitate, the mother fluid was distilled in vacuo to 1/3 the volume and stood again in the refrigerator overnight. The deposit hereby was put together with the former, washed with 5 cc. of a 3% solution of crystalline barium acetate several times and suspended in 10 cc. of water. The suspension was acidified to pH 1.2 with 10% HCl and shaken with 20 cc. of chloroform continuously for 12 hours. The watery layer which separated on standing was extracted with two more changes of chloroform in a similar manner, and the chloroformic solutions combined were washed three

times with an equal volume of water, distilled under reduced pressure and desiccated in vacuo over H_2SO_4. A yellowish non-sticky powder weighing 160 mg. was yielded—Lipid AO_1 (Lipid AO after the first run). The procedure above was repeated five times more with new batches of material and 820 mg. of Lipid AO was obtained in total.

2) Pig lung group lipid mixture (*Lipid BO*). Masamune and Hirata's procedure[2] was somewhat modified. Pig lungs were freed from blood vessels and bronchi and masticated. The parencyhma brei was dehydrated by standing in 5 volumes of acetone for 10 days and extracted with hot alcohol (94%). The hot alcoholic solutions were united and cooled, and the part that precipitated in the cold was removed. The solution was then distilled in vacuo to syrupy consistency, freed from the acetone-soluble and ether- and benzene-insoluble parts and treated with a mixture of dry ether and dry alcohol in the volume proportion of 1:8 in turn. The part remaining undissolved in the ether-alcohol mixture was washed with acetone and shaken with benzene after dissolving in slightly alkaline water. The watery solution was acidified and again shaken with benzene. The part which passed into benzene at this stage was further fractioned with chloroform after taking up in acidulated water and the chloroform-soluble division separated was washed with acetone and dried. The product (Lipid BO) from fresh lungs of 8 pigs (4585 g.) amounted to 850 mg.

The anti-hemagglutinative potencies of the preparations are illustrated in Table I (A, B).

Preparation of Antisera

1) Anti-A serum. 5 cc. portions of 10% suspensions in physiological saline of human Group A erythrocytes were intravenously injected into a rabbit at intervals of 2 days, and after the tenth injection, the animal was bled to separate the serum, which was inactivated at 56°C for 30 minutes. 5 cc. of the serum was then diluted with an equal volume of physiological saline, 10 cc. of washed human B erythrocytes was added, and after shaking, the mixture was kept at 37°C for 2 hours and subsequently at 0°C for 5 hours. The B erythrocytes were centrifuged off at 2000 rpm for 5 minutes. The absorption was repeated in a similar manner until the serum was almost no more agglutinative to B erythrocytes. The anti-A serum thus prepared had anti-A agglutinin titer of 1024. It scarcely agglutinated either of B and O erythrocytes.

2) Anti-B serum. Ten 3 cc. portions of 10% suspensions of human B erythrocytes were injected into a chicken similar to above. The anti-B serum absorbed with human A erythrocytes was capable of agglutinating A and O erythrocytes only faintly. It had anti-B agglutinin titer of 1024.

3) Anti-O serum. A chicken was immunized with ten 3 cc. portions of human OS saliva and the antiserum was absorbed with human Os saliva. It was almost unable to agglutinate A and B erythrocytes. Anti-O alggutinin titer 1640.

Results of assays for agglutinin potencies of these antisera are detailed in Table II.

TABLE I

Anti-hemagglutinative Potencies of Lipids AO, BO, A, B and O

The assay was processed in test-tubes as described by Masamune *et al.*,[18] but on a smaller scale in the cases of Lipids A and O. AO_1, A_1, O_1, AO_5, A_6 and O_6 signify preparations of respective Lipids after the first, fifth or sixth run. E: eel serum. G: normal anti-O goat serum. OS: anti-OS saliva chicken serum. −: no agglutination. ±: faint aggl. (ascertainable by the aid of a magnifier). +: slight aggl. (observable with the naked eye). ++: marked aggl.

A. Detailed illustration of assay with respect to examples picked out

Lipid	Red cells	Serum	Dilution of the antigens (n in $1:100\times2^n$)												
			0	1	2	3	4	5	6	7	8	9	10	11	12	∞
AO_1	A	α	−	−	−	−	−	−	−	−	−	−	−	±	+	++
	B	β	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	O	Anti-E	−	−	−	−	−	−	−	±	+	+	++	++	++	++
	O	G	−	−	−	−	−	−	−	−	±	+	+	++	++	++
	O	Anti-OS	−	−	−	−	−	−	−	+	+	+	++	++	++	++
BO	A	α	+	+	++	++	++	++	++	++						++
	B	β	−	−	−	−	−	−	+	+						++
	O	Anti-E	−	−	±	+	+	++	++	++						++
	O	G	−	−	+	+	+	++	++	++						++
	O	Anti-OS	−	−	+	+	+	++	++	++						++
A_1	A	α		−	−	−	−	−	−	−	−	−	−	−	+	++
	B	β		++	++	++	++	++	++	++	++	++	++	++	++	++
	O	Anti-E		±	+	+	+	++	++	++	++	++	++	++	++	++
	O	G		−	+	+	+	+	+	+	++	++	++	++	++	++
	O	Anti-OS		−	+	+	+	+	++	++	++	++	++	++	++	++
B	A	α	+	+	++	++	++	++	++	++	++	++	++			++
	B	β	−	−	−	−	−	−	−	+	+	+	++			++
	O	Anti-E	+	+	+	+	++	++	++	++	++	++	++			++
	O	G	±	+	+	+	++	++	++	++	++	++	++			++
	O	Anti-OS	+	+	+	++	++	++	++	++	++	++	++			++
O_1	A	α		−	±	±	+	+	+	+	++	++	++	++	++	++
	B	β		++	++	++	++	++	++	++	++	++	++	++	++	++
	O	Anti-E		−	−	−	−	−	−	−	−	−	±	+	+	++
	O	G		−	−	−	−	−	−	−	−	−	±	+	+	++
	O	Anti-OS		−	−	−	−	−	−	−	−	±	+	+	++	++

B. Group active (anti-hemagglutinative) dilutions

Red cells		A	B	O		
Serum		α	β	Anti-E	G	Anti-OS
Lipid	AO_{1-6}	$1:100\times2^{9-10}$	$1:<100$	$1:100\times2^{4-9}$	$1:100\times2^{4-9}$	$1:100\times2^{4-9}$
	BO	$1:<100$	$1:100\times2^5$	$1:100\times2$	$1:100\times2$	$1:100\times2$
	A_{1-6}	$1:100\times2^{9-11}$	$1:<100\times2$	$1:100\times2^{1-3}$	$1:<100\times2\sim1:100\times2^3$	$1:<100\times2\sim1:100\times2^3$
	B	$1:<100$	$1:100\times2^6$	$1:<100$	$1:<100$	$1:<100$
	O_{1-6}	$1:<100\times2\sim1:100\times2^5$	$1:<100\times2$	$1:100\times2^{7-9}$	$1:100\times2^{6-9}$	$1:100\times2^{6-9}$

TABLE II

Agglutinative Potencies to Red Cells of the Anti-A, Anti-B and Anti-O Sera

1 drop of the original and progressively diluted antisera were mixed together with 1 drop of 1% suspensions of A, B or O erythrocytes on a slide and stood at room temperature (12–15°C) for 30 minutes to judge the occurrence of hemagglutination. − : no agglutination. ± : faint aggl. + : slight aggl. ++ : marked aggl.

Serum	Red cells	Dilution of the sera													
		n in $1:2^n$													
		0	1	2	3	4	5	6	7	8	9	10	11	12	∞
Anti-A	A	++	++	++	++	++	++	++	++	+	+	+	−	−	−
	B	±	−	−	−	−	−	−	−	−	−	−	−	−	−
	O	±	−	−	−	−	−	−	−	−	−	−	−	−	−
		n in $1:2^n$													
		0	1	2	3	4	5	6	7	8	9	10	11	12	∞
Anti-B	A	±	−	−	−	−	−	−	−	−	−	−	−	−	−
	B	++	++	++	++	++	++	++	++	+	+	+	−	−	−
	O	±	−	−	−	−	−	−	−	−	−	−	−	−	−
		1:1	n in $1:5\times2^n$												
			0	1	2	3	4	5	6	7	8	9	10	∞	
Anti-O	A	±	−	−	−	−	−	−	−	−	−	−	−	−	
	B	±	−	−	−	−	−	−	−	−	−	−	−	−	
	O	++	++	++	++	++	++	+	+	+	−	−	−	−	

Isolation of Individual Group-specific Lipids from Lipids AO_1 and BO by Precipitin Reaction

I) Precipitin test was effected by combining the antigen-dilution and antibody-dilution methods in 1×5 cm. test tubes. Lipids AO_1 and BO were dissolved in 100 and 20 volumes of saline respectively and progressively diluted with saline. 0.3 cc. each of the original and diluted solutions of the antigens and of those of the antisera were mixed together and maintained at 15°C for 5 hours, during which the occurrence of precipitate was judged by means of a magnifier from time to time. As shown in Table III, the antigen : antiserum ratios at which the reaction took place most quickly (optimum ratios) were $1 : 500\times(4/3)^8 \sim 4 : 500\times(4/3)^8 \fallingdotseq 1 : 1250$ and $1 : 100\times(4/3)^2 \sim 2 : 100\times(4/3)^5 \fallingdotseq 1 : 200$ between the stomach group lipid mixture and anti-A serum and between the same lipid mixture and anti-O serum respectively. The corresponding ratio between the lung group lipid mixture and anti-B serum was $2^2 : 40\times(4/3)^4 \sim 2^2 : 40\times$

Table III

Precipitin Reaction of Lipids AO and BO with Antisera

Regarding the method of assay, see the text. Lipid AO_1 signifies the preparation of Lipid AO after the first run. − : no reaction. ± : faint reaction. + : slight reaction (A very thin layer of precipitate was seen at the bottom of test tube). ++ : marked reaction.

Antigen	Antiserum and its dilution		Dilution of the antigens													
			n in $1:500\times(4/3)^n$													
			0	1	2	3	4	5	6	7	8	9	10	11	12	13
Lipid AO_1	Anti-A serum	n in $1:2^n$ 0	+	+	+	+*	+	+	+	+	+	+	±	±	±	±
		1	±	±	±	±	+	+	+*	+	+	−	±	±	±	±
		2	−	−	−	−	±	±	±	±	±*	±	±	±	±	±
		3†	−	−	−	−	−	−	−	−	±	±	±	±	±	±
		4	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		∞	−	−	−	−	−	−	−	−	−	−	−	−	−	−
			n in $1:100\times(4/3)^n$													
			0	1	2	3	4	5	6	7	8	9	10	11	12	13
	Anti-O serum	n in $1:2^n$ 0	+	+	+*	+	+	+	+	±	±	±	−	−	−	−
		1	±	±	±	±	+	+*	+	±	±	±	−	−	−	−
		2†	−	−	−	−	−	±	±	±	±	±	−	−	−	−
		3	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		∞	−	−	−	−	−	−	−	−	−	−	−	−	−	−
			n in $1:40\times(4/3)^n$													
			0	1	2	3	4	5	6	7	8	9	10	11	12	13
Lipid BO	Anti-B serum	n in $1:2^n$ 0†	++	++	++	+	+	+	+	+	+	±	±	±	−	−
		1†	++	++	+	+	+	+	+	+	±	±	±	±	−	−
		2	+	+	+	+	+*	+	+	+	±	±	±	±	−	−
		3	±	±	±	+	+	+	+*	+	±	±	±	±	−	−
		4†	−	−	−	−	−	−	−	±	±	±	±	±	−	−
		5†	−	−	−	−	−	−	−	−	−	−	±	±	−	−
		∞	−	−	−	−	−	−	−	−	−	−	−	−	−	−
			n in $1:20\times(4/3)^n$													
			0	1	2	3	4	5	6	7	8	9	10	11	12	13
	Anti-O serum	n in $1:2^n$ 0	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		1	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		2	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		3	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		4	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		∞	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* Signifies the dilution of antigen where precipitin reaction occurred most quickly in the respective range of antigen-dilution. † It was difficult to discern the optimum antigen-antiserum ratio.

$(4/3)^4 \fallingdotseq 1:30$. Between the last lipid mixture and anti-O serum no precipitate appeared. In each range of antigen dilution in the table, the most voluminous precipitate was given in the test-tube next or next but one to the left to that which contained the antigen and antiserum in the optimum ratio.

2) In order to ascertain whether or not any component other than the

antibodies, which precipitates Lipid AO and/or BO partially at least, is present in the antisera, solutions of Lipid AO_1 ($1:30\times2^{0-14}$) or of Lipid BO ($1:20\times2^{0-10}$) were laid over the anti-A and anti-O sera completely absorbed with A erythrocytes and OS saliva respectively or over the anti-B serum completely absorbed with B erythrocytes, followed by standing at 10–13°C for 5 hours. No precipitates occurred at all.

3) Group A-specific lipid (Lipid A). Lipid AO_1 and the anti-A serum were mixed in the proportion of 1:700 to obtain the maximum amount of precipitate, namely, 10 cc. of the antiserum was poured into a stoppered glass tube of 2.5×7 cm., made up to 15 cc. with saline and cooled at 0°C for 6 hours, and 2 cc. of saline containing 15 mg. of the antigen (an opalescent solution) similarly chilled was added. (The diluted antiserum and antigen solution both gave no precipitate, when chilled.) The mixture was placed in a water-thermostat at 37°C for 2 hours with occasional shaking and then in the refrigerator (0°C) for 1 week. In the control without the antigen which was carried out on a small scale, no precipitate was given at all, but, in the main run, a pretty large precipitate settled within the first 1 day. The precipitate was centrifuged off at 6000 rpm for 15 minutes in a refrigerated centrifuge (0°C), and the supernatant was set aside—Fr. Ia_1. The centrifugate was washed with 3 cc. portions of saline at 0°C and shaken with 4 cc. of 15% NaCl at 12°C for 1 hour. After standing in the refrigerator for 5 hours, the insoluble part was separated by centrifugation at 0°C. The supernatant fluid here was dialyzed against changes of saline in the refrigerator for 2 days, and some deposit that occurred during the dialysis was rejected after filtration. The filtrate was distilled at low temperature ($<$ 20°C) to 0.5 cc. and preserved in the refrigerator (Fr. Ib_1). The insoluble part in 15% NaCl above was dissolved in 0.2 cc. of 0.01 *N* NaOH, 20 cc. of acetone was added and the precipitate was centrifuged off and placed in a desiccator containing H_2SO_4. The substance dried (10–15 mg., biuret +) was boiled with four 3 cc. portions of dry benzene under reflux, each extraction taking 5 minutes and being followed by filtering while hot. The solutions were combined, washed with an equal volume of water three times and concentrated in vacuo at 25–30°C to dryness. The still-residue was well dried in a vacuum desiccator (H_2SO_4) overnight, taken up in 3 cc. of dry benzene and after discarding the tiny insoluble part, again distilled in vacuo to dryness with succeeding placement in the desiccator—Lipid A_1. Yield 4.4 mg.

4) Group B-specific lipid (Lipid B). 250 mg. of the group-lipid mixture from pig lungs was dissolved in 15 cc. of saline. The opalescent solution was chilled (It gave no precipitate) and added to 5 cc. of the anti-B serum, previously chilled, under agitation (antigen-antiserum ratio 1:20). The mixture was kept at 37°C for 2 hours and at 0°C for 1 week with subsequent centrifugation (The control gave no precipitate). The supernatant (Fr. IIa) was set aside, and the centrifugate was washed with five 5 cc. portions of chilled saline and shaken with 15 cc. of 10% NaCl at room temperature (12°C) for 1 hour. The centrifuged supernatant here was dialyzed against saline at 0°C and concentrated in vacuo to 0.5 cc. (Fr. IIb). The insoluble part in 10% NaCl was dissolved in

0.8 cc. of 0.01 *N* NaOH, diluted with 30 cc. of saline, added to with 5 g. of NaCl and acidified to pH 1.2 by the aid of 1 *N* HCl (The solution changed slightly turbid) in turn. It was shaken with 30 cc. of chloroform at 37°C for 15 minutes. The chloroformic layer after centrifugation was drawn out, and the watery and gel layers were subjected together to 5 repetitions of similar extraction, using 30 cc. of chloroform for each extraction. The chloroformic solutions were united, neutralized to pH 7.0 with 0.1 *N* NaOH and distilled at 25°C to about 50 cc. It was next washed with an equal volume of water four times and distilled in vacuo to dryness. The still-residue was taken up in 10 cc. of water, acidified to pH 1.2 with 0.1 *N* HCl and again shaken with 10 cc. of chloroform. The centrifuged watery layer was exhausted twice more as above. The chloroformic solutions at this stage were washed well with water and distilled under reduced pressure to dryness. Yield 54 mg.

5) Group O-specific lipid (Lipid O). 100 mg. of Lipid AO_1 was dissolved into an opalescent solution in 5 cc. of saline, chilled (No precipitate occurred) and added to 10 cc. of the anti-O serum also chilled (antigen-antiserum ratio 1:100) and the mixture was kept at 37°C for 2 hours and at 0°C for 1 week. The precipitate (In the control processed without Lipid AO_1 no precipitate appeared) was centrifuged. The supernatant (Fr. $IIIa_1$) was set aside, and the centrifugate was washed thoroughly with 3 cc. portions of saline at 0°C and shaken with 5 cc. of 15% NaCl at 15°C for 1 hour. The centrifuged supernatant here was dialyzed against changes of saline and distilled to 0.5 cc. similar as in *4)* (Fr. $IIIb_1$). The part insoluble in 15% NaCl was dissolved in 0.2 cc. of 0.01 *N* NaOH, 20 cc. of dry acetone was added, and the deposit was dried and exhausted with 3 cc. portions of benzene as in *3)*. The solutions given were combined, washed with an equal volume of water three times and concentrated to dryness also as in *3)*. The still-residue was taken up in 2 cc. of dry benzene, the very small insoluble part was centrifuged off and discarded, and the supernatant was condensed in vacuo until dryness—Lipid O_1. 5.3 mg. was the yield.

Yields of the Group A- and Group O-specific lipids were so small that preparation of them was run several times, one specimen each of them being prepared from each of the successive materials Lipids AO_1–AO_6.

Regarding the anti-hemagglutinative potencies of the preparations of the group-specific Lipids A, B and O, see Table I. The group activity of the preparations of Lipids A and O paralleled A and O activities respectively of their preparation materials Lipid AO and pig stomach mucosa, which paralleled each other on their side regarding those activities.

6) Hemagglutinative and anti-hemagglutinative potencies of Frs. Ia, IIa and IIIa and hemagglutinative potencies of Frs. Ib, IIb and IIIb. The fractions (fluids) were assayed regarding their hemagglutinative potency as were the antisera (Rf. to the explanatory note of Table II). Frs. Ia_1, IIa and $IIIa_1$ did not agglutinate erythrocytes of all the groups at all, indicating that no excessive antibody remained in the mother fluids of the antigen-antibody precipitates, whereas they contained excess antigen as shown in Tiable IV. Frs. Ib, IIb and IIIb proved strongly agglutinative to Group A, B and O erythrocytes respectively (Table V).

Table IV

Anti-hemagglutinative Potencies of Frs. Ia, IIa and IIIa (Fluids)

The assay was effected according to Masamune *et al.*[18] Frs. Ia_1 and $IIIa_1$ mean the respective fractions in the first runs. Anti-E: eel serum. G: normal anti-O goat serum. Anti-OS: Anti-OS saliva chicken serum. −: no agglutination. ±: faint aggl. +: slight aggl. ++: marked aggl.

Fr.	Red cells	Serum	Dilution of Frs. (n in $1:2^n$)													
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	∞
Ia_1	A	α	−	−	−	−	−	−	+	+	+	++	++	++	++	++	++
	O	Anti-E	−	−	−	±	+	+	+	++	++	++	++	++	++	++	++
	O	G	−	−	−	−	+	+	+	+	++	++	++	++	++	++	++
	O	Anti-OS	−	−	−	+	+	++	++	++	++	++	++	++	++	++	++
IIa	B	β	−	−	−	±	+	+	+	+	+	++	++	++	++	++	++
	O	Anti-E	−	−	±	+	+	+	+	++	++	++	++	++	++	++	++
	O	G	−	−	±	+	+	+	+	+	+	+	+	++	++	++	++
	O	Anti-OS	−	±	+	+	+	+	++	++	++	++	++	++	++	++	++
$IIIa_1$	A	α	−	−	−	−	−	−	−	−	−	−	±	+	+	+	++
	O	Anti-E	−	−	−	−	−	+	+	+	+	+	++	++	++	++	++
	O	G	−	−	−	−	−	−	−	+	+	+	++	++	++	++	++
	O	Anti-OS	−	−	−	−	−	±	+	+	+	+	+	++	++	++	++

Table V

Hemagglutinative Dilution of Frs. Ib, IIb and IIIb (Fluids)

Regarding the method of assay see the text. Frs. Ib_1, $IIIb_1$, Ib_6 and $IIIb_6$ signify the respective fractions in the first or sixth runs of preparing the group-specific Lipids.

Red cells		A	B	O
Fr.	Ib_{1-6}	$1:2^{8-11}$	$1:<1$	$1:<1$
	IIb	$1:<1$	$1:2^{7-10}$	$1:<1$
	$IIIb_{1-6}$	$1:<1$	$1:<1$	$1:2^{7-9}$

Properties of the Group Lipid Mixtures and Individual Group-specific Lipids

The balances of the corresponding preparations of Lipids AO, A or O were mixed together for the physical and chemical examinations.

1) Solubility. The solubility of Lipids AO, BO, A, B and O are detailed in Table VI, indicating that no alteration of solubility occurred by the serological fractionation.

2) Electrophoresis. The substances all showed one symmetrical boundary after electrophoresis and that with nearly the same mobilities between the group-specific lipids and their respective lipid materials from which they had been separated (Fig. 1).

3) Test-tube tests. All the substances gave similar results: Molisch and

TABLE VI

Solubility of Lipids AO, A, O, BO and B

AO, A and O are mixtures of the balances after the serological assays of the preparations of the respective Lipids. − : insoluble. ± : faintly sol. + : partly sol. ++ : mostly sol. ### : completely sol.

Lipid	Solvent															
	Ether		Petrol ether	Methanol		Ethanol		Benzene		Chloroform		Water				
												20°C			70°	
	20°C	boiling	20° boiling	20°	50°	20°	50°	20°	50°	20°	50°	pH 1–5*	6.4	8–10†	pH 1–5*	6.4 8–10†
AO	−	−	− −	−	±	−	−	###	###	###	###	±	+	###	+	++ ###
A	−	−	− −	−	±	−	−	###	###	###	###	±	+	###	+	++ ###
O	−	−	− −	−	±	−	−	###	###	###	###	±	+	###	+	++ ###
BO	−	−	− −	±	−	−	+	###	###	###	###	±	+	###	±	++ ###
B	−	−	− −	±	−	−	+	###	###	###	###	±	+	###	±	++ ###

* Acidified with HCl. † Alkalinized with NaOH.

Rosenthaler (for alcohol) positive; biuret and direct as well as indirect Osaki-Turumi negative.

4) Paper-chromatographic assays. The procedure of Masamune *et al.*[14] was adapted as follows. a) Water-insoluble components. 15 mg. substance was heated with a mixture of 1.5 cc. each of dry methanol and conc. HCl (sp. gr. 1.18) under reflux at 100°C in a 2.5×11 cm. tube for 6 hours, and the hydrolysate was centrifuged when cold. The centrifugate was washed with 2 cc. of dry methanol. The supernatant fluid and washing were combined and energeti cally shaken with five 10 cc. portions of petrol ether. The petrol-ethereal extracts were united and washed several times with an equal volume of water until they turned neutral, and distilled in vacuo to dryness. The still-residues at this stage of the procedure started with the various lipid mixtures and group specific lipids were colored brown to yellowish brown and gelatinous. Yielded was 1.9 mg. from Lipid AO, 3.5 mg. from Lipid BO, 2.2 mg. from Lipid A, 3.7 mg. from Lipid B and 2.1 mg. from Lipid O. The products showed the positive Feigl reaction for carboxyl (processed without treatment with $SOCl_2$) and the positive Rosenthaler reaction for alcohol.

Of each of their 0.5% petrol-ethereal solutions three 0.03 cc. portions were sampled separately on 2.5×60 cm. slivers of Toyo Roshi filter paper No. 2, which were irrigated with "Solvent III" (butanol satd. with water-phenol satd. with water-ethanol mixture in volume proportions of 5:2:1.5) in the ascending way. In each set of the chromatograms one was sprayed with ninhydrin in butanol sat. with water and alkaline permanganate reagents in turn, but no

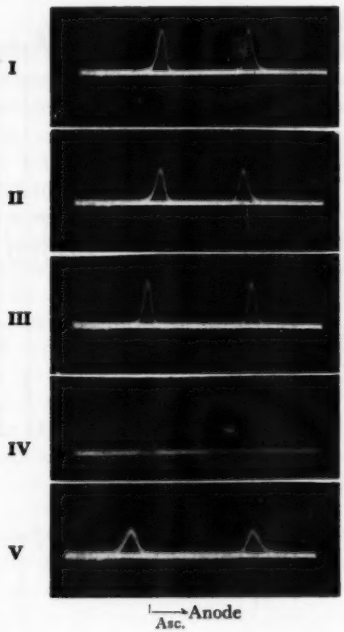

Fig. 1. Electrophoretic patterns (4/5×) of 0.5% (w/v) solutions of Lipid AO (**I**), A (**II**), O (**III**), BO (**IV**) and B (**V**) in phosphate buffer pH 7.8, I 0.29. Temperature of test 10°C in **I**, **II** and **IV** and 7°C in **III** and **V**. Exposure 60 minutes in **I**, **II** and **III** and 30 minutes in **IV** and **V** after starting current. Mobility :

$$\text{Lipid AO}: \frac{(-1.25)\times 0.0118\times 0.3}{0.01\times 3600} = -12.3 \text{ cm}^2/\text{volt/sec}\times 10^{-5}$$

$$\text{A}: \frac{(-1.26)\times 0.0115\times 0.3}{0.01\times 3600} = -12.1 \quad "$$

$$\text{O}: \frac{(-1.19)\times 0.0117\times 0.3}{0.01\times 3600} = -11.6 \quad "$$

$$\text{BO}: \frac{(-1.11)\times 0.0094\times 0.3}{0.01\times 1800} = -17.4 \quad "$$

$$\text{B}: \frac{(-1.03)\times 0.0094\times 0.3}{0.01\times 1800} = -16.2 \quad "$$

sphingosine spot was indicated for any of the lipid mixtures and lipids. Second chromatograms sprayed with Schiff reagent showed no spot of aldehyde. Third chromatograms were sprayed with the methylred reagent. Here was exhibited a diffuse, comparatively strong pink spot in the cases of Lipid BO and Lipid B and a weaker spot in the cases of Lipid AO and Lipids A and O, suggesting a fatty acid or acids.

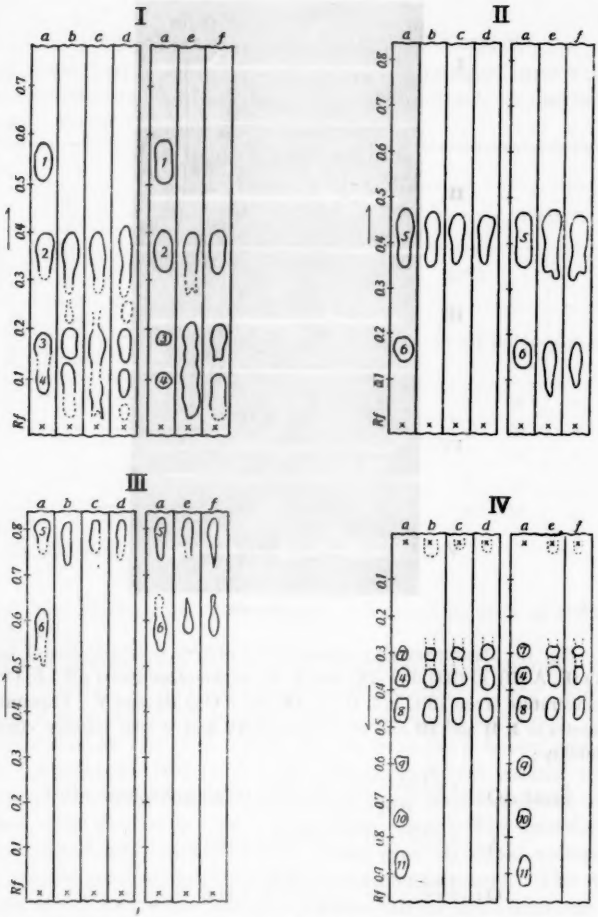

Fig. 2. Chromatograms of the water-soluble components after methanolysis (**I–III**) and hydrolysis (**IV**) of Lipids AO, A, O, BO and B. Temperature of test 24°±1°C. **I**: First stage chromatograms developed by ascending irrigation with Solvent II. Solvent fronts run 45 cm. Spots indicated with iodine and ninhydrin in turn. **II** and **III**: Fourth stage chromatograms developed by ascending irrigation with Solvent IV (**II**) and V (**III**). Solvent fronts run 45 cm. Indicator lead acetate-H_2S. **IV**: Third stage chromatograms developed with Solvent butanol-pyridine-water (5:3:2) by descending irrigation. Duration of test 24 hrs. Indicator aniline hydrogen phthalate reagent of Partridge.

Reference runs were carried out by applying 0.02 cc. of standard solutions which were 1% with respect to individual solutes.

b) Water-soluble components (Fig. 2 and 3). i) The methanol-conc. HCl layer after extraction with petrol ether in a) was distilled under reduced pressure at 25–30°C to about 0.5 cc. and repeatedly evaporated to a thick solution in a vacuum desiccator containing solid caustic soda, adding water from time to time. When the solution became less acidic than pH 2.0, it was dried up, and the remainder was dissolved in 0.1 cc. of water, followed by careful addition of 0.1 *N* NaOH to pH 6.8.

First stage chromatography. Three 0.02 cc. portions of each of the solutions given were sampled on paper slivers same as the ones in a) and one of the slivers was irrigated with "Solvent I" (butanol satd. with water-phenol satd. with water-ethanol mixture in volume proportions of 5:3:1) in an ammonia-containing atmosphere and the other two slivers with "Solvent II" (butanol sat. with water-phenol satd. with water-glacial acetic acid mixture in volume proportions 5:2:1). One of the chromatograms developed with Solvent II was sprayed at first with 0.5% iodine in 95% alcohol, but no spot corresponding to choline was detected in all the cases of the lipid mixtures and group-specific lipids. The chromatogram was freed from iodine by hanging in air and thereafter sprayed with ninhydrin, whereby spots of ethanolamine, hexosamine and glycine and/or serine were indicated for any of the substances examined (Fig. 2I). The remaining two chromatograms were tested with Dragendorff reagent for choline with negative result for any of the substances.

Second stage chromatography. An 0.03 cc. portion of each of the solutions was sampled on a 30×30 cm. square of Toyo Roshi filter paper of the same number as above and submitted to two-dimensional chromatography by irrigating the square with 80% phenol (an ammonia-containing atmosphere) primarily and with a butanol-acetic acid-water mixture in the volume proportions of 4:2:1 secondarily. The chromatogram was sprayed with the ninhydrin reagent above for indication. As shown in Fig. 3 (I–V), aspartic and glutamic acids, serine, glycine, alanine, arginine, hexosamine, ethanolamine and valine and/or methionine were detected for Lipids AO, A and O, and the same components exclusive of valine and/or methionine for Lipids BO and B.

Fourth stage chromatography. Two paper-slivers were spotted with each of the solutions as in First stage, and they were irrigated one with "Solvent IV" (80% ethanol) and the other with "Solvent V" (ethanol-methanol-glycerol-water mixture in volume proportions of 2:1:1:2) in the ascending

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a: reference run. *b*: Lipid AO. *c*: Lipid A. *d*: Lipid O. *e*: Lipid BO. *f*: Lipid B. *1* choline, *2* ethanolamine, *3* glycine, *4* glucosamine hydrochloride, *5* glycerophosphoric acid, *6* inositolphosphoric acid, *7* galactosamine hydrochloride, *8* galactose, *9* mannose, *10* L-fucose, *11* rhamnose. (Rf values of choline are 0.63 and 0.55 respectively when it is chromatographed in the ascending way with Solvents I and II, and those of glycerophosphoric and inositolphosphoric acids are 0.44 (A diffuse spot extends over Rf 0.37–0.50) and 0.20 (A diffuse spot extends over Rf 0.13–0.27) respectively in ascending chromatography with Solvent IV and 0.80 and 0.60 respectively in ascending chromatography with Solvent V.)

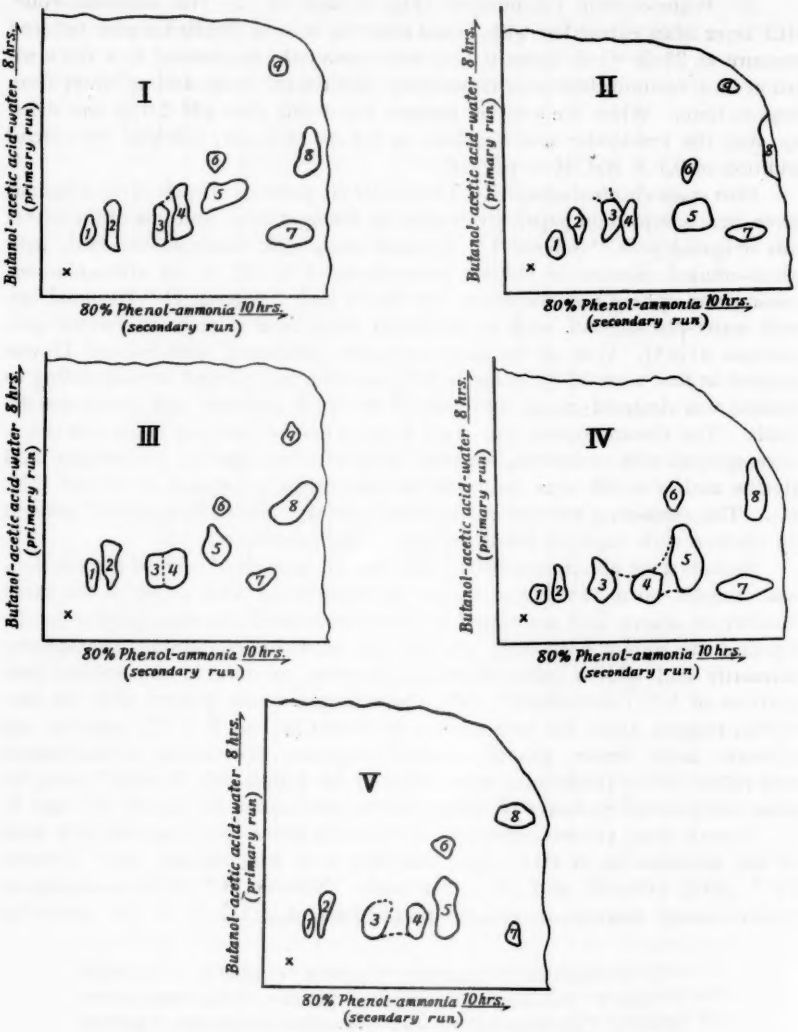

Fig. 3. Second stage chromatograms (two dimensional, each irrigation being ascending) of the water-soluble components of Lipids AO (**I**), A (**II**), O (**III**), BO (**IV**) and B (**V**). Temperature of test 22°±1°C. Indicator ninhydrin.

1 aspartic acid, *2* glutamic acid, *3* serine, *4* glycine, *5* a hexosamine or hexosamines, *6* alanine, *7* arginine, *8* ethanolamine, *9* valine and/or methionine.

way, impregnated with 5% neutral lead acetate, and after washing with water and drying, exposed to H_2S gas. One spot only was shown at the position corresponding to glycerophosphoric acid in the cases of Lipids AO, A and O, but two spots at the positions corresponding to glycerophosphoric acid and inositolphosphoric acid (weak) in the cases of Lipids BO and B (Fig. 2II and 2III).

ii) Paper-chromatography of sugar components (Fig. 2IV). The third stage chromatography was carried out on new hydrolysates as follows. 5 mg. substance was sealed with 1 cc. of 1 *N* H_2SO_4 in an ampoule and heated at 100°C for 5 hours. The humin was removed after filtration, and the filtrate was neutralized to pH 6.8 with a 10% solution of baryta ($Ba(OH)_2 \cdot 8H_2O$) and centrifuged. The supernatant and the washing of the centrifugate were distilled under reduced pressure to about 0.1 cc., and an 0.03 cc. portion of the condensate was applied on a 7.5×60 cm. sliver of Toyo Roshi filter paper No. 2. Descending irrigation was effected with a mixture of butanol, pyridine and water in the volume proportions of 5:3:2. The indicator used was the aniline hydrogen phthalate reagent of Partridge. Spots of galactosamine, glucosamine and galactose were shown in all the cases of the Lipids.

5) Quantitative-analytical figures are embodied in Table VII. It is to be remarked that the analytical figures of group-specific Lipids A and O and of Lipid B resemble closely those of Lipid AO and of Lipid BO respectively.

TABLE VII

Composition of Lipids AO, A, O, BO and B

AO, A and O were mixtures of the balances after the serological assay of the preparations of the respective Lipids.

Analysis	In per cent					In equivalents per equivalent weight				
	AO	A	O	BO	B	AO	A	O	BO	B
N*	2.29	2.30	2.32	2.36	2.38					
P†	2.16	2.12	2.17	4.07	4.18	1.73	1.71	1.74	38.5	38.6
Hexosamines as glucosamine‡	7.20	7.16	7.22	0.61	0.62	1.0	1.0	1.0	1.0	1.0
Galactose§	7.45	7.42	7.56	1.25	1.28	1.03	1.03	1.04	2.04	2.05
Ash//	2.4	3.2	1.9	4.2	3.8					

* Micro Kjeldahl. † Haneda, Sinokawa and Yasuoka's modification[20] of Plimmer method. ‡ Masamune and Yosizawa.[21] § Masamune and Sakamoto.[22] // Pregl method without use of H_2SO_4.

The group-specific Lipids A and O are similar to Lipid AO and Lipid B to Lipid BO with respect to solubility and electrophoretic mobility. Furthermore, Lipids A and O resemble Lipid AO and Lipid B resembles Lipid BO not only qualitative- but also quantitative-analytically in so far as was examined. It is no wonder that the group-specific lipids from the same tissue source and their mixture are composed alike, when analogized from the similarity of the

group mucopolysaccharides A, B and O from one source in respect to quantitative composition excepting glucosamine/galactosamine ratio and contents of a few other components which are absent in the group lipids.[15)-19)] It is hardly ascribed to contamination that the lipid moiety fully accompanies the non-lipid moiety in the specific precipitin reaction. The fact rather furnishes a firm evidence for the chemical entity of the group lipids in addition to those mentioned in the introductory note.

Summary

1. A group lipid mixture AO (Lipid AO) was prepared from pig stomachs and a mixture BO (Lipid BO) from pig lungs, and group-specific lipids A and O (Lipid A and Lipid O) were isolated from the former mixture by the aid of anti-A and anti-O sera and group-specific lipid B (Lipid B) from the latter by the aid of an anti-B serum.

2. Lipids AO, BO, A, O and B proved all electrophoretically homogeneous. The boundaries of the group-specific lipids (A, O and B) migrated with mobilities practically identical with those of their respective materials Lipids AO and BO.

3. Lipids A and O had the same solubility as Lipid AO, and Lipid B the same solubility as Lipid BO.

4. Test-tube tests resulted same among the substances.

5. The group-specific lipids A, O and B proved to be made up of the same components qualitatively as well as quantitatively as their respective materials (AO and BO) from which they had been separated, in so far as was examined.

6. These findings affirm our belief that those group-specific lipids are chemical entities but not mixtures of lipid and group mucopolysaccharide. In addition, the individual occurrence of substances A, B and O but not of substances AO and BO has been established concerning the group lipids of the present kind.

The Ministry of Education gave a grant in aid to us through the Grant Committee for Scientific Researches, which is gratefully acknowledged. H. Masamune.

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A Physical Consideration on the Nakazawa-Nakai's Density-Meter

By

Shigetomi Nakai, Haruhiko Hayashi
(中井 重宝) (林 晴彦)
and **Gen-ichiro Arakawa**
(荒川 源一郎)
From the Medical Clinic of Prof. F. Nakazawa, Tohoku University, Sendai

and

Hirofumi Fujimoto and **Takesi Hayasi**
(藤本 弘文) (林 威)
From the Physical Institute, Faculty of Science, Tohoku University, Sendai

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In a previous paper one of the authors (S. N.) described the measurements of the specific gravity of biological fluids by the dilution method due to Nakazawa and Nakai, and it is found that the Nakazawa-Nakai's density-meter is of very practical value owing to its simplicity and accuracy. To develop the method of the N-N's density-meter further, its physical nature was investigated by comparing with the weighing method for measurement of the density of liquid.

1) Weighing Method for Measuring the Density of Liquid, and the Density of Pure Water

For precision-measurement of the density of liquid we prefer the standard method of weighing a solid body in the air and in the liquid. The accuracy of the method will be ascertained by measuring the density of pure water.

The density S of a solid body and the density Q of water are related by the following equation[1]:

$$S=\frac{m}{w}Q+(1-\frac{m}{w})\lambda \qquad (1)$$

where λ means the density of air, which can be assumed as 0.00120 g/cm^3 in most cases, m the weight in the air of the solid body, w the weight in the air of water, having an absolute density Q, of the same volume as that of the solid body. Eq. (1) shows that the density of water Q, or liquid in

general, can be obtained, if the density S of the solid body is known. This method is used for the precision-measurement of the density of liquid in the following.

As a solid body we took a piece of fused quartz-glass in the form of a right circular cylinder of the following size, of which the lengths at 20°C were measured by a millimeter compared with the standard block gauges:

Mean value of diameters:	18.9463 mm.
Mean value of sectional areas:	2.819803 cm^2.
Mean value of heights:	27.6520 mm.
Volume:	$V=7.797311$ cm^3. at 20°C,

where the volume was very carefully calculated by graphical integration as a sum of partial volumes of several layers. With an analytical balance of sensitivity 1 mg. the weight of the cylinder was measured;

Weight in the air: $m=17.16838$ g.

The mass of the cylinder m_0 can be obtained, correcting the bouyancy by air for the weights used and the cylinder, as

$$m_0=17.17552 \text{ g.}$$

Then the density S of the cylinder is calculated:

Density: $S=m_0/V=2.20275$ g/cm^3 at 20°C.

The weighing of the cylinder in pure water gave

$$w=7.77226 \text{ g.}$$

Then the density of the pure water is calculated by Eq. (1):

$$Q=0.99801 \text{ g/cm}^3 \text{ at } 19.5°\text{C},$$

which is smaller than the corresponding value 0.998305 g/cm^3 given by Kohlrausch[2]. The difference of 0.0003 g/cm^3 may be caused by the difficulty of measuring the volume of the cylinder or by the impurity of the water used, or to some other cause. For the accuracy 0.001 g/cm^3 requested in practice, the above mentioned difference of the absolute density is negligibly small.

2) Physical Nature of the Floating Sphere Used in N-N's Density-Meter

a) *Sensitivity for the density of liquid.* An aqueous solution of NaCl was prepared, in which the small floating sphere of the density-meter was kept in equilibrium, i.e. without floating up or down. The floating sphere used in this experiment is the same as that used by Nakai in his previous investigation.[3] The density of the solution was measured by the method mentioned in paragraph 1); considering Q in Eq. (1) as the density of the solution, we obtained the density Q 1.00072 g/cm^3 at 15°C, for the floating sphere to show equilibrium. Then small grains of NaCl were added to the solution and thus we had a solution of the density 1.00095

g/cm³, in which the floating sphere had an upward velocity of 0.87 mm/sec at 15°C. In the other solution of NaCl of the density 1.00049 g/cm³ at 15°C the floating sphere had a downward velocity of the same magnitude as the above. The velocity of the sphere 1.74 mm/sec was caused by

TABLE I

Density d_t

t°C	d_t g/cm³
6.2	1.00079
7.2	79
13.6	73
14.5	69
23.7	61
28.5	51
31.2	42
36.0	34

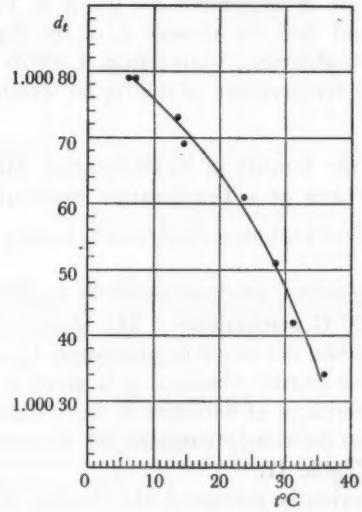

Fig. 1. Density d_t of the liquid, in which the floating sphere is kept in equilibrium.

the difference 0.00046 g/cm³ in density. Therefore, the difference of 0.0001 g/cm³ caused the floating velocity of 0.38 mm/sec, which was easily recognizable with the eye. Thus the sensitivity of the density-

	Density g/cm³	
A:	1.00075	at 10°C,
B:	1.00071	at 15°C,
C:	1.00048	at 30°C,

meter is sufficient for the purpose of measuring the density of blood.

b) *Effect of atmospheric pressure.* The density-meter was put in a vessel, in which the pressure was controlled by a pump. The velocity of the floating sphere rising or falling in the solution at 18°C was repeatedly measured under different atmospheric pressures: 656, 756 and 856 mm. Hg. Almost no difference of velocity was recognizable, at most the difference was only 1 mm/min. The effect of the difference in atmospheric pressure can practically be ignored, and a detailed description of the experiment may be unnecessary.

c) *Effect of temperature of solution.* By the method described in paragraph 1), the density d_t of the NaCl solution, in which the floating sphere was kept in equilibrium at t°C, was measured for the temperature range $10 \leqq t \leqq 30$. Then the density of the quartz-glass cylinder, which had the density $S=2.20275$ g/cm^3 at 20°C, was corrected for each temperature t°C, assuming the linear expansion-coefficient 6×10^{-7} for quartz-glass.

The results of the measurement are given in Table I and shown in Fig. 1. It was found that the density d_t of the liquid, which kept the floating sphere in equilibrium, varied from 1.00076 to 1.00048 g/cm^3, in accordance with the temperature of the liquid which was varied from 10 to 30°C.

3) Comparison of the Density of NaCl-Solution with That of a Mixture of Blood and Water at a Temperature between 10–30°C

Three kinds of NaCl solution A, B, and C having the following density were prepared:
in which the floating sphere was kept nearly in equilibrium at the temperatures 10, 15 and 30°C respectively. The density of each solution was measured by the method described in paragraph 1) over the temperature-range 10–30°C. The density obtained q_t is given in Table I and shown in Fig. 2. The differences of densities of the solutions (A and B) or (A and C) are found to be nearly constant for the temperature range 10–30°C, as shown in Table II.

Nakai[3] has previously measured the density of a mixture of blood and water, in which the floating sphere was kept in equilibrium at 15°C, as a function of temperature over the range 10–30°C. He used the specific gravity bottle of the volume 10.265 cc. The weight of both the bottle and the mixture filled in it is taken from his measured values, and are given in the second column of Table III. The difference between the weight measured at t°C and that measured at 15°C, divided by the volume of the bottle, gives the difference in density at t°C as referred to the standard density at 15°C. The density of the mixture at 15°C is given as 1.00067 by our present experiment in paragraph 2)–c). The density of the mixture

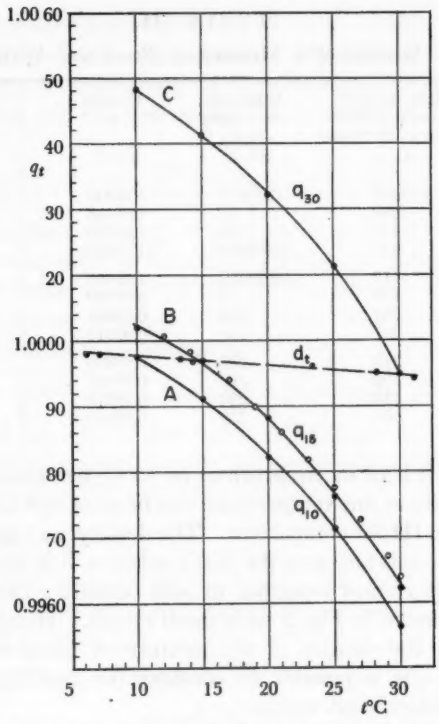

Fig. 2. Density q_t of NaCl-solution, in which the floating sphere is kept in equilibrium at t°C the d_t-curve is taken from Fig. 1. Small circles marked along the q_{15} curve represent the density of the mixture of blood and water measured by Nakai. Cf. Paragraph 3).

TABLE II

Density of NaCl-Solutions and Differences

t°C	Density q_t g/cm³			Difference in density	
	A	B	C	B–A	C–A
10	1.00075	1.00122	1.00482	0.00047	0.00407
15	1.00012	1.00072	1.00412	60	400
20	0.99922	0.99982	1.00322	60	400
25	0.99813	0.99862	1.00212	49	399
30	0.99662	0.99722	1.00050	60	388

d_t at each temperature can be calculated and is shown in the fourth column of Table III.

On the other hand, the density of NaCl solution B, in which the

TABLE III

Density of a Mixture of Blood and Water

t°C	Weight of both bottle and mixture measured by Nakai g	Difference in density d_t-d_{15} g/cm³	Density d_t g/cm³	Density of NaCl solution B q_{15} g/cm³
10	40.9494	0.00051	1.00120	1.00122
12	480	37	1.00106	1.00105
14	456	14	1.00083	1.00074
15	442	[0.00000]	[1.00069]	1.00072
17	412	−0.00029	1.00040	1.00040
19	370	− 70	0.99999	1.00000
21	331	− 108	0.99961	0.99962
23	287	− 150	0.99919	0.99915
25	243	− 193	0.99876	0.99870
27	193	− 242	0.99827	0.99815
29	136	− 298	0.99771	0.99760
30	104	− 329	0.99740	0.99730

floating sphere is kept in equilibrium at 15°C, are shown in Fig. 2, from which the density at any temperature can be read and are given in the fifth column of Table III for comparison. The density, as a function of temperature, of both the mixture and the NaCl solution B is almost the same and the maximum-deviation amounts to only 0.00012 g/cm³ The density of the mixture is shown in Fig. 2 with small circles. Hence, so far as we are concerned with the density of the mixture of blood and water at any temperature t°C, it is possible to consider the NaCl solution instead of the mixture of blood and water.

4) Method of the Nakazawa-Nakai's Density-Meter.

The method of N-N's density-meter is a dilution method and will be illustrated by a measurement of the density of blood. Let us take the blood of volume 0.4 cc. and of density ρ_t at t°C. Pure water of volume v and of density Q_t at t°C is added to it, so that the floating sphere remains in equilibrium in the mixture of blood and water at t°C, where the mixture has the density d_t given in paragraph 2)-c). If the additivity of volumes of blood and water is valid, then $v=V_t-0.4$, where V_t means the total volume of blood and water. The mass of the mixture is given by $0.4\,\rho_t+vQ_t$ or as well as by V_td_t. Therefore the mass relation is expressed by

$$0.4\rho_t+vQ_t=V_td_t \qquad (2)$$

For the volumetry of blood 0.4 cc., of water v and of the mixture V_t, it is necessary to assume that the pipetts used for measuring these volumes have the same thermal expansion and are calibrated at the same temperature, e.g. t°C. Then Eq. (2) can hold as an equation between the scale-readings on the pipetts used at t°C. Hence we have, making use of $v=V_t\sim0.4$, from Eq. (2)

$$\rho_t = Q_t + 2.5\ V_t(d_t - Q_t) \qquad (3)$$

A scale marked on the density-meter corresponds to every 0.2 cc. The scale reading s corresponding to V_t indicates the sum of volumes of the mixture V_t and the floating sphere c: $0.2\ s = V_t + c$. Then the Eq. (3) becomes

$$\rho_t = Q_t + \frac{1}{2}s(d_t - Q_t) - \frac{5}{2}c(d_t - Q_t). \qquad (3a)$$

Assuming that the density Q_t of pure water at t°C is the same as given by Kohlrausch,[2] and making use of the density d_t as measured in paragraph 2)-c) and of the volume of the floating sphere $c = 0.0171$ cc., corresponding to the sphere of radius 1.6 mm., the numerical values of ρ_t can be obtained as a function of temperature t and scale division s.

The last term $\frac{5}{2}c(d_t - Q_t)$ in Eq. (3a) represents the effect of the volume of the sphere and has the largest value 0.00020 for 30°C, which is less than the limit 0.001 requested for the measurement of density of blood in most cases. Neglecting the last term, we have

$$\rho_t = Q_t + \frac{1}{2}s(d_t - Q_t) \qquad (3b)$$

The density of blood ρ_t is calculated by Eq. (3b) and is listed in Table IV, (in which the last term of Eq. (3a) is also included), and ρ_t is shown graphically in Fig. 3a and 3b.

5) Reduction of Density of Blood at 15°C from the Scale-Division Measured at t°C

The method of N-N's density-meter measures generally the volume of the mixture of blood and water at t°C (other than 15°C), in which the floating sphere is kept in equilibrium. The volume thus measured depends upon the temperature t of the mixture. If we assume the temperature 15°C as the standard temperature for the density of blood, then it is of practical value to obtain the density of blood at 15°C directly from the reading of the volume measured at t°C, for which Nakai has given the reduction table.[3] Under the same idea, the reduction table will be recalculated in the following, by taking the dependency of various quantities upon temperature into account.

Suppose that the mixture at t°C ($t > 15$) has the reading of volume V_t and the density d_t, to keep the floating sphere in equilibrium at t°C. If the mixture is cooled to 15°C, then it has the density x and the reading of volume V_{15}. Then we have the mass of the mixture

$$d_t\ V_t\ (1 + \beta(t - t_0)) = x\ V_{15}(1 + \beta(15 - t_0)) \qquad (4)$$

where β means thermal expansion coefficient of the glass of the density-meter, which can be assumed as 0.000024, and t_0 is the temperature, at

TABLE IV

(a) d_t Q_t as a Function of Temperature t

	t°C				
	10	15	20	25	30
d_t	1.00075	1.00070	1.00064	1.00056	1.00047
Q_t	0.99973	0.99913	0.99823	0.99707	0.99567
d_t-Q_t	0.00102	0.00157	0.00241	0.00349	0.00480
$\frac{5}{2}c(d_t-Q_t)$	0.00004	0.00007	0.00010	0.00015	0.00020

(b) Density of Blood $\rho_t=Q_t+\frac{1}{2}s(d_t-Q_t)$ as a Function of s and t

s	t°C				
	10	15	30	25	30
10	1.0048	1.0070	1.0103	1.0145	1.0197
20	1.0099	1.0148	1.0223	1.0320	1.0437
30	1.0150	1.0227	1.0344	1.0494	1.0677
40	1.0201	1.0305	1.0464	1·0669	1.0917
50	1.0252	1.0384	1.0585	1.0843	1.1157
60	1.0303	1.0462	1.0705	1.1018	1.1397
70	1.0354	1.0541	1.0826	1.1192	1.1637
80	1.0405	1.0619	1.0946	1.1367	1.1877
90	1.0456	1.0698	1.1067	1.1542	1.2117
100	1.0507	1.0776	1.1187	1.1716	1.2357

which the volume-scale gives the correct value of the volume.

To find the floating sphere in equilibrium in the mixture at 15°C, its density must be d_{15}. To get the density d_{15} of the mixture one must add an adequate amount of water at 15°C to the mixture and then the total volume of the mixture is read as v_{15}. The mass of the added water is given by

$$Q_{15}(v_{15}-V_{15})\ (1+\beta(15-t_0),$$

where Q_{15} denotes the density of water $Q_{15}=0.999126$ at 15°C. Then we have for the mass of the mixture

$$d_tV_t(1+\beta(t-t_0))+Q_{15}(v_{15}-V_{15})(1+\beta(15-t_0))=d_{15}v_{15}(1+\beta(15-t_0)) \quad (5)$$

Eliminating V_{15} from Eq. (4) and (5), and neglecting higher powers of β, it becomes

$$d_tV_t(1+\beta(t-15))\ (1-\frac{Q_{15}}{x})/(d_{15}-Q_{15})=v_{15} \quad (6)$$

If we put $V_t=\frac{1}{5}s_t-c$; $V_{15}=\frac{1}{5}s_{15}-c$, where s_t means the reading of

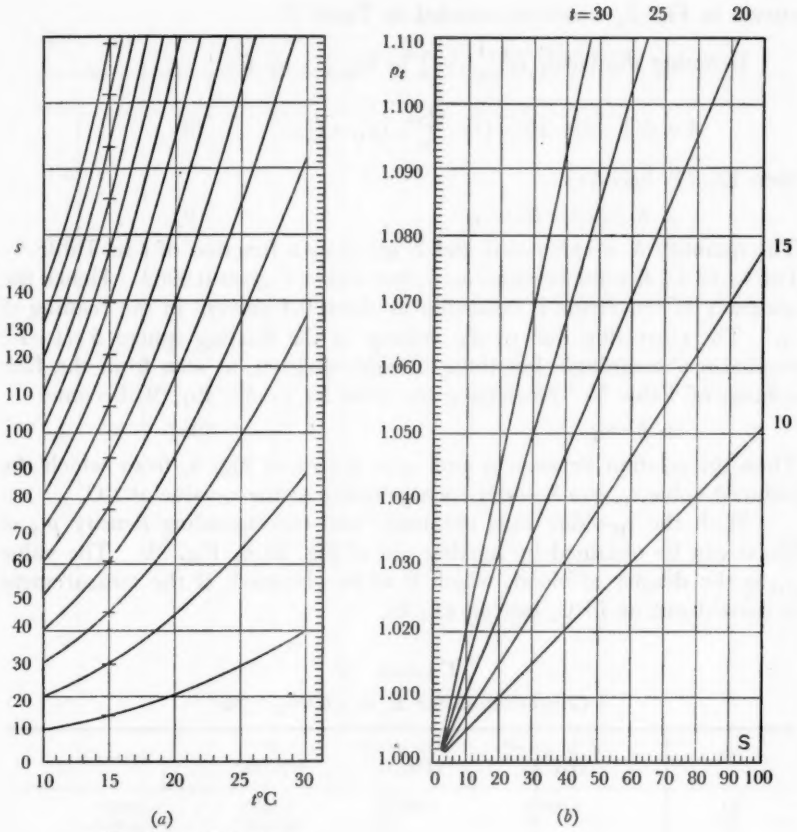

Fig. 3. ρ_t (s, t)—curves.

the density-meter corresponding to V_t, and c the volume of the floating sphere $c=0.0171$ cc., then Eq. (6) becomes

$$d_t(\frac{1}{5}s_t-c)\ (1+\beta(t-15))\ (1-\frac{Q_{15}}{x})/(d_{15}-Q_{15})=\frac{1}{5}s_{15}-c \qquad (7)$$

which gives the reduced reading s_{15} corresponding to the reading s_t measured at t°C.

The quantity x in Eq. (7) means the density of mixture at 15°C, when it is cooled to 15°C from its initial temperature t°C. The measurement of x was carried out by Nakai[3] for the mixture which had the density d_{15} at 15°C, but the equal density of the mixture and of the NaCl solution found in paragraph 3) and the parallelism of q_t-curves with the q_{15}-curve in Fig. 2 give the quantity x from the differences between d_t- and q_{15}-

curves in Fig. 2, which is included in Table *V*.

Denoting the factor of $(\frac{1}{5}s_t - c)$ in Eq. (7) by K:

$$K = d_t(1+\beta(t-15))\ (1-\frac{Q_{15}}{x})/(d_{15}-Q_{15}) \qquad (8)$$

then Eq. (7) becomes

$$s_t\ K + 5c(1-K) = s_{15} \qquad (9)$$

The quantity K is calculated and is given as a function of t in Table V. For $t=15°C$, K must be equal to 1, but Table *V* gives 0.9962. Hence the accuracy of conversion is estimated as about 0.4 percent in the reading of s_{15}. The correction due to the volume of the floating sphere $5\,c(1-K)$ results in a magnitude less than 0.2 scale-division, as seen from the fifth column of Table V. Neglecting the term $5\,c(1-K)$, Eq. (9) becomes

$$s_t\ K = s_{15} \qquad (9a)$$

Then the relation between s_t and s_{15} is shown in Fig. 4, from which the reduced value s_{15} can be read corresponding to the s_t-value at $t°C$.

With the s_{15}-value thus obtained, the corresponding density ρ_{15} of blood can be obtained by making use of Fig. 3a or Fig. 3b. The value ρ_{15} is the density of blood, which is to be obtained, if the measurement is carried out at 15°C, instead of $t°C$.

TABLE V

Conversion-factor K in $s_tK = s_{15}$ (9a)

t°C	d_t in g/cm³ (Fig. 1)	x in g/cm³ (Fig. 2)	K Eq. (9a)	$5c(1-K)$
10	1.00076	1.00015	0.6526	0.0297
12	73	30	0.7482	0.0215
14	71	54	0.9012	0.0084
15	70	69	0.9962	0.0003
16	69	87	1.1109	−0.0095
18	,66	117	1.2824	−0.0241
20	64	150	1.5113	−0.0437
22	61	190	1.7650	−0.0654
24	58	234	2.0443	−0.0892
25	56	250	2.1456	−0.0979
26	55	269	2.2661	−0.1082
28	51	319	2.5830	−0.1353
30	47	376	2.9432	−0.1661

SUMMARY

1. By the method of weighing given in paragraph 1), investigations were made on the physical nature of the floating sphere used in the N-N's density-meter: on the sensitivity for the density, on the effect of variation

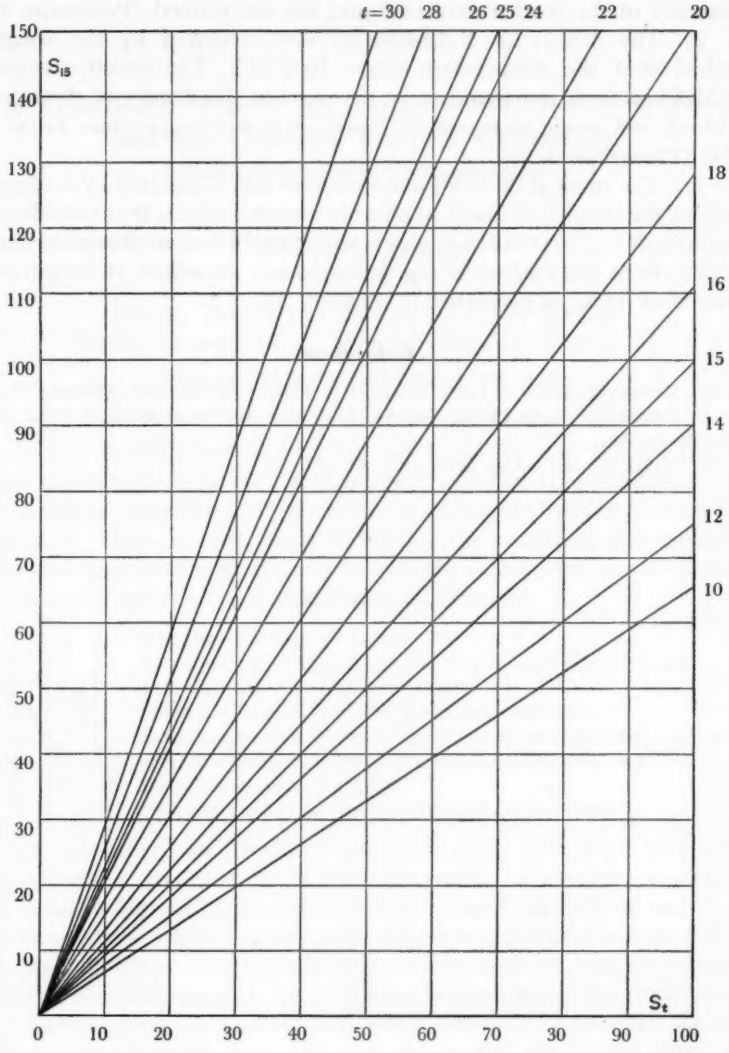

Fig. 4. $s_t K = s_{15}$

of the atmospheric pressure and on the effect of variation of the temperature of liquid. It is proved that the N-N's density-meter is of high sensitivity, which allows one to measure the difference of density 0.0001 g/cm³ of liquid easily, provided that high purity of water used for dilution and the

constancy of the temperature of liquid are ascertained (Paragraph 2)).

2. The density of NaCl-solution was measured by the weighing method over the temperature range 10–30°C. The density measured for NaCl-solution was found to be the same as the density of the mixture of blood and water measured by Nakai, over the temperature range 10–30°C (Paragraph 3)).

3. The method of N-N's density-meter was elucidated by a measurement of the density of blood, taking the thermal effects into consideration (Paragraph 4)). A reduction chart, which enables us to obtain the density at 15°C from the reading of the density-meter measured at temperatures other than 15°C, is presented (Paragraph 5)).

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A Continued Report on Reticulo-endothelial System aud the Changes Thereof Caused by Inflammatory Stimulation

By

Kaneyoshi Akazaki
(赤崎 兼義)

From the Department of Pathology, School of Medicine
Tohoku University, Sendai. Director: Prof. Dr. K. Akazaki

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Past Views Concerning Reticulo-endothelial System

It was Aschoff and his collaborator Landau[1] that the specific cell group the author deals with in this report was first consolidated in 1913 into a system, and named "Milzapparat oder endothelialer Stoffwechselapparat." Then in 1924, Aschoff[2] taking the results of the researches both inside and outside his country into consideration, renamed it "Reticulo-endothelial system," and classified it as follows:

R.E.S. in a wider sense
- R.E.S. in a narrow sense
  - 1) Reticulum cells of pulp of spleen
    Reticulum cells of cortical nodules and pulp cords of lymph nodes
    Reticulum cells of other lymphatic tissues
  - 2) Reticulo-endothelia of sinus of spleen and lymph nodes
    Reticulo-endothelia of capillaries of liver lobules (Kupffer's stellate cells), bone marrow, adrenal cortex and pituitary anterior lobe
- 3) Histiocytes in connective tissues
- 4) Splenocytes and phagocytic monocytes (blood histiocytes)

On the other hand, Kiyono[3] named in 1920 Aschoff's "Das reticulo-endotheliale System" histiocytic cell-system. According to him, the cells constituting the mentioned cell system are 1) histiocytes and 2) reticulo-endothelia which may be subdivided into (a) reticulum cells and (b) reticulo-endothelia. He regards those three kinds of cells as essentially identical, the difference of the cell form depending on the difference of their environment. He stated that reticulo-endothelia too take the form of histiocyte when set free. That is, he unified the above three into "histiocytic cell-system" because of their identity in their histiocytic features. However, Aschoff remarked that it is not yet certain as to whether those cells are completely identical or not is to be decided in future.

At any rate, reticulo-endothelial system is a name originally given to a group of cells with same phagocytic tendency, and the classification has

been solely based on the result of the tests concerning dye-storing activity by means of vital staining methods. However, later development of pathological investigations on that cell system; especially accumulation of the knowledge concerning the tumors originating in the cell system excited various doubts about the claims of Aschoff and Kiyono, and various opinions have been expressed to improve them and the system was variously named.

For instance, the so-called "Das retotheliale System" of Rössle[4] and his pupil Roulet[5] signifies what Aschoff and Kiyono called Retikulum-Zellen, excluding their Reticulo-endothelien as a different kind of cell. The reason for their renaming "Retikulum-Zellen" "Retothelien" is that those cells adhere to argyrophil-fiber-net and that they wanted to classify them against Endothel. Consequently, it is no other than "Retikulum-Zellen-System" or only another name for it. It is to be added that the authors disregarded the problem of histiocytes.

Then Rohr[6] used also the name "Retikulo-histiozytäres System," but he meant in it a system including Aschoff's reticulo-endothelial system in a wider sense fibrocytes, plasma cells, mast cells, giant cells and the alike. There are not a few different opinions and names for the cell system in question, but it has little connection with what the writer is going to talk with.

What impresses us in reviewing both the theories of Aschoff and of Kiyono and those advanced later on, is that the category of the cells belonging to that system differs according to investigator, because the past study on the cells constituting the system was restricted mostly to histological investigations by vital staining method without sufficient embryological and cytological study in parallel with it. Consequently it is natural that the differentiation of histiocytes from monocytes should become extremely difficult if, as some recent investigators (who consider macrophages in the peritoneal cavity to be monocytes) assert, such strong phagocytic ability as that of histiocytes is attributed to monocyts, which is denied by Aschoff and Kiyono.

What makes the solution of this problem more difficult is that the form of the cells belonging to the system changes markedly on slightest stimulation, as will be stated below. For instance, the inflammation of skin often induces the appearance of small round atypical histiocytes, but the differentiation of such cells is very difficult. Probably they have often been disposed of simply as lymphocytes, monocytes, or plasma cells. In short, present situation does not allow any orientation concerning this cell system at all.

Investigation on Reticulo-endothelial System

Such being the circumstances we[7] attempted an investigation on the

cells belonging to the system from as many angles as possible chiefly by cytological method, aiming at an ideal classification. As to the differentiation among similar cells, it was[8] endeavoured first to elucidate, that the cells of this system not only show marked variance according to the different types but also can show amazing transformation after various stimulations including the physiological. In addition, supravital staining of subcutaneous histiocytes and Giemsa staining (Hirata-Fujii's[9] method) of stretch preparations gave always very satisfactory result, as will be briefly described below.

A. Relation between reticulo-endothelial cells and argyrophil fibres

According to our results,[10] cells what arouse question in the system are, as Kiyono pointed out, reticulum cells, histiocytes, and reticulo-endothelia. For the classification of those cells, one cannot ignor their relation with argyrophil fibers.

First as to reticulum cells, they are those which form reticulum with argyrophil fibers. With regard to the relation between such reticulum cells and argyrophil fibers, there have been put forward varied views. Of German scholars in particular, many seem to have considered that argyrophil fibers themselves are produced by reticulum cells as their protoplasmic projections. Fresen,[11] who is commonly accepted as the greatest German authority at present in study along this line, also is an advocator of the theory of argyrophil fiber formation by reticulum cells. Our investigation[12] led us to conclude that argyrophil fibers are formed independently from the cells. The tissue portions of fiber formation are not always the areas where reticulum cells are present. For instance, in the dermic layer where reticulum cells are originally absent and where the fibrous structure is chiefly of cellagenous fibers, perfect argyrophil fiber network of lattice-like structure is formed whenever infiltration of lymphosarcoma cells occurs (the same is true with lymphatic leukemia). When argyrophil fiber network is once formed, reticulum cells adhere to it more readily than any other cells probably due to the special nature of their protoplasm. Argyrophil fibers are essentially identical with collagenous fibers, as is evident from their distinct mutual transition. This fact has also been verified by the result of Irvine and Toulis' electron-microscopic investigation.[13]

It seems that the relation between histiocytes and argyrophil fibers has been discussed very little. According to the result of our investigation,[14] those cells are usually found in the places where the so-called reticular fibers are absent such as skin. If argyrophil fibers are formed, histiocytes adhere to them and turn to reticulum cells. This has been established[14] through findings in both human and rabbit's lamina pro-

pria mucosae of small intestine.

Then as for the relationship between reticulo-endothelia[15] and argyrophil fibers, it is evident that those cells adhere also to the inner side of the argyrophil fibers in capillary cleft and sinusoidal wall, but as will be stated below, this kind of cell possesses very strong phagocytic function in distinction from endothelial cells, both of which we often detect in separated syncytial form. Thus a quite unique relation exists between reticuloendothelia and argyrophil fibers and distinguishes the former cells form reticulum cells. Viewed embryologically,[16] the cells in the bone marrow of the embryo of the 3rd month for instance which will later represent reticulo-endothelia of the sinusoidal wall take already the definite form of endothelia but not the primitive form of reticulum cells.

B. Vital staining findings of reticulo-endothelial cells

Vital staining has been a useful method for determining the cell classification under the said cell system ever since Aschoff and Kiyono, and the findings thereby may be said to indispensable for their investigations. In our Department, Onishi[17),18),19),20)] has made a thorough investigation concerning this method. Apart from detailed discussion, it may be worthy to point out or three items which are significant in connection with the present study. It has been made clear that the findings of vital staining should not be hastily judged, unless the conditions have been sufficiently analyzed because the experimental results widely vary depending on the size of the dye granules used as well as on the frequently of the dye administration. To describe in detail the vital staining dye-stuff, which is intravenously injected, is taken up first and most easily in reticulo-endothelia owing to the positional relation. Reticulum cells and histiocytes store smaller amounts of the dye-stuff than reticulo-endothelia unless the dye is injected in sufficient amount and frequency. Although such dyes with high diffusibility as trypan-blue or Evans-blue are stored fairly well by reticulum cells and histiocytes even in the case with small dose of injection.

But caution must be taken in the use of those dyes since they are strongly poisonous. When the amount and frequency of the dye injections reach beyond a certain degree, reticulum cells and histiocytes show their vital staining figures more distinctly than reticulo-endothelia, Kupffer's stellate cells excepted. This is quite conceivable when it is taken into consideration that the neutral-red vacuoles, which are the vital stain-storing apparatus, are more vividly developed in reticulum cells and histiocytes than in general reticulo-endothelia.

With the intravenous injection of Chinese ink, whose dye granules are coarse and which has extremely weak tissue-diffusibility, the particles

are chiefly stored by reticulo-endothelia. In order to obtain the dye-storing figures of reticulum cells and histiocytes, it is necessary ro repeat the injection of Chinese ink at least 30 or more times according to Sugiyama.

Thus it is necessary to examine various conditions to make any judgement about the result of vital staining. It is important to correctly select the dye-stuff to be used, since most of them have beneficial and faulty properties for the present purpose. According to Onishi,[17] the ability for storing vital staining dye-stuff is jedged most appropriately if 2.5% lithium-carmine solution is injected once a day in a dose of 3.6–3.8 cc. per kg. body weight for a period of 5–7 days. Blood monocytes[21] also possess the same ability, but it is far less marked as compared with that of reticulo-endothelia.

As is evident from the above fact, it should be remarked that it is extremely difficult to determine the distribution and the range of reticulo-endothelial cells simply from the findings of vital staining; and that even if one fills reticulo-endothelial cells with such a substance as Chines ink granules for instance, it blocks only a part of the cells of the said systeme.

C. Cytological investigation on reticulo-endothelial cell

It is evident from what stated above that examination on impregnated preparation and vital staining figures alone cannot be sufficient to determine the range of the cell groups belonging to reticulo-endothelial system. Therefore, it was tried by us[22] to grasp the specific features of the cells of the said system by cytological procedures. For this purpose, we applied supravital staining with neutral-red and Janus-green, May-Giemsa's staining of stamp Preparation, detection of Golgi apparatus, oxidase and peroxidase test, and Kon's silver test; and we found that the first two methods in particular furnish very unique findings.

First, by the aid of supravital staining, the mitochondria of reticulum cells were well distinguished which are densely distributed around nuclei but sparcely near the rim of cell bodies. The neutral-red vacuoles differ in figure according to the degree of the cell differentiation, but in those of the characteristic and mature type, the vacuoles stainable in orange color and of varied size filled cell bodies.

As to histiocytes we examined chiefly those of subcutaneous portions. The findings thereof were generally the same as those of reticulum cells. Only it was found that generally in histiocytes the neutral-red vacuoles are very small. This is what inferred from the vital stainings, because it is not easy for the stimulation by blood circulation to reach the said cells. When stimulated in any way, histiocytes change their structure so as to resemble reticulum cells.

As to reticulo-endothelia, they are discriminated from reticulum cells

and histiocytes, except for Kupffer's stellate cells, by the facts that the mitochrondria are found near the both ends of nucleus, which are more or less distorted; and the neutral-red vacuoles are fewer and smaller, although Kupffer's stellate cells are difficult to distinguish from them when their neutral-red vacuoles increase in number and size. In May-Giemsa's stain preparation, reticulum cells and histiocytes look very much alike, most of them being irregular in the form of cell body and some round. Their cell bodies are often indistinct, the protoplasm being stained slightly basophil, sometimes with azurophil granules or unstainable vacuoles, or with phagocyted foreign bodies. The nuclei were mostly round or oval or lobulated but those constricted in the middle were also occasionally found among young cells and among the cells under stimulation. In short, the nuclear structure is characteristic as described above, whereas the cell bodies of reticulo-endothelia are of spindle shape—except for the stellate cells functionally accelerated—and their long elliptical nuclei have usually one or several longitudinal winkles and occasionally are constricted or screwed, with the chromatin nets stained more deeply than those of reticulum cells. With regard to the findings on Golgi apparatus, oxidase and peroxidase reactions and Kon's silver reaction, all three of them were the same.

Judging from the above result of cytological investigations, reticulum cells and reticulo-endothelia show slight but definite different figures despide that reticulum cells and histiocytes are of structures very much alike. It must be admitted here that such reticulo-endothelia with highly accelerated function as typical Kupffer's stellate cells for instance can be hardly distinguished from reticulum cells even by those method of cytological examination above.

D. Varied forms of histocyte.

a) *The abnormal figures of cutaneous histocytes after stimulations.* The cells of reticulo-endothelial system show extremely varied figures upon various stimulations. Although the mode of such changes varies according to the intensity, the kinds as well as the process of stimulation, some common features are discerned among them.

For instance, when 10 cc. of typhoid vaccine are injected into peritoneal cavity of a rabbit of about 3 kg. body weight, the cells of reticulo-endothelial system of the whole body are activated, as it were, and begin to proliferate. In such a case, the skin is most convenient as the object of investigation to see the process of the changes in histiocytes because there is no emigration of leucocytes. Even at the third day after the injection, histiocytes are seen proliferating a characteristic way—some in island form and some others in cord from. In such proliferating histiocytes, there are seen not

only the large ones which are commonly found but also small round ones in many places. Supravital staining exhibits in those round cells a number of mitochondria of short rod form or fine granular form with the neutral-red vacuoles clustering one side of the nuclei or being sometimes arranged in rosettes. The May-Giemsa's preparation of such cell groups demonstrates round or oblong nuclei or those with monocyte-like compressions; some others are often found located in a side of the cell body reminding the one in plasma cells. These cells have strongly basophilic cell protoplasm. The cell bodies of such cells are rather thin, and they are mixed with the cells resembling lymphocytes. They are probably what are called lymphocytes, small monocytes, plasma cells etc. with reference to ordinary stained preparations; but supravital staining reveals them to be histiocytes in disguise.

Then in the experiment of subcutaneous autoblood injection on rabbits, the same histological reaction as that caused by typhoid vaccine injection—except for slight leucocytic infiltration—was observed. That is, in the subcutaneous tissues, histiocytes were seen proliferating as soon as 30 minutes after the injection and some are jointed together in cords and some are isolated and take round cell form. Three days later, some small round histiocytes, which had markedly proliferated, were seen acting as erythrophages. Further, in the experiment with cutaneous injection of agar and fine powdered bone to rabbits, the same nest-form proliferation of histiocytes as was observed in the above experiment occurred; and in such cases, the histiocytes were seen to have turned into such small round cells as closely resemble lymphocytes, monocytes, and plasma cells.

Among such proliferating histiocytes are those which gradually swell to become like epitheloid cells or turn into Langhans' giant cells. Thus it is evidently concluded that histiocytes react to the slightest stimulation and proliferate and further that they turn to small round cells, or may take the form of lymphocytes, monocytes, or plasma cells, or may turn to epitheloid cells and Langhans' giant cells. It is to be specially mentioned that many of those cells show the so-called rosette formation of the neutral-red vacuoles or lobular nuclei.

The figures of such histiocytes in disguise as were found in subcutaneous tissues are also detectable among histiocytes under normal conditions. What are called the macrophages in peritoneal cavity are an example of such normal histiocytes.

b) Nature of macrophages of peritoneal cavity. As is well known, a number of isolated round macrophages are present in the intrapritoneal fluids physiologically. Recently an increasing number of investigators have regarded this kind of cell as monocytes because of their round and isolated form, their lobular nuclei, their neutral-red vacuoles as revealed

by supravital staining, and their positive peroxidase reaction. The size of this kind of cell range from that of lymphocytes to that six times as large as neutrophile leucocytes, thus making the conception of them as blood monocytes inapplicable even from their size. Furthermore, histiocytes under stimulation are also inclined to grow round and become isolated, the neutral-red vacuoles often showing rosette formation and the nuclei lobular or constricted form.

Bearing these questions in mind, Murata[23] in our Department made a detailed investigation to get an accurate knowledge concerning the nature of macrophages in the peritoneal cavity. First of all, this kind of cell proved outstanding in its strong phagocytic tendency—stronger than that of monocytes and equalling that of histiocytes—May-Giemsa's staining figures of the nuclei more closely resembling those of monocytes. The greatest question here is that those cells are said to give positive peroxidase reaction. Except in rabbits, histiocytes are generally peroxidase negative, whereas monocytes are peroxidase positive. Hence, Murata examined the perioxidase reaction of the cells under varied conditions, and verified that they really show the negative reaction. Those,[24] who asserted the tendency of the cells of giving the positive result in peroxidase reaction, injected intraperitoneally a peptone solution in order to easily extract the peritoneal fluid. This stimulation caused migration of a large number of neutrophile leucocytes into the fluid, whose cell bodies contain the substances showing positive peroxidase reaction. Taking in those neutrophile leucocytes by phagocytosis, the macrophages in view turned positive in the reaction.

From the above facts, it is evident that the varied features of peritoneal macrophages closely resemble those of the histiocytes in inflammatory foci, which makes it permissible to regard them as histiocytes. By a later investigation on such macrophages in our Department, the presence of such macrophages not only in the peritoneal cavity but also in pleural, pericardial and joint cavities has been confirmed. The question still lies in the mystery as to what physiological stimulation constantly maintains histiocytes in such a stimulated state. Experiments have been carried out to elucidate it without any conclusive result as yet.

E. Embryological investigation on subcutaneous histiocytes

In most cases, histiocytes and fibroblasts are difficult to distinguish from each other in stained preparations unless the former show marked phagocytic figures. Under a cytological examination they not only show different vital staining figures but possess different nuclear structures and forms of cellular granules, although there exist sometimes cells which are difficultly differentiated. This may be the reason for the existence of the

assumption affirming the transition between those two kinds.

Next, with the purpose of elucidating the relation between the two in the prenatal stage and also to study the cytological findings of young histiocytes, both embryos and babies of rabbits were examined. The subjects of our examination were 11 embryos ranging from the 10th day of pregnancy up to the time of delivery; and 15 baby rabbits from the time of delivery up to the 10th day. A cytological examination of their subcutaneous tissue was made in the stretched preparations stained both by supravital staining and with May-Giemsa's staining.

It was found that some of mesenchymal cells begin to show the chracteristics of histiocytes earlier than expected. In the subcutaneous tissue of an 10 days old embryo, some cells of histiocytic appearance were already present either scattered or in groupings by twos and threes among the cells with fibroblastic chracteristics. Those cells show the cytological properties distinctly differeing from those of urfibroblasts, and the nuclei are richly chromatic when stained according to May and Giemsa. They are smaller in size, unique in form, and resemble subcutaneous histiocytes of matured rabbits in supravital staining, only differing in one respect, that is, the neutral red vacuoles are often arranged in rosette form.

Those cells already show high phagocytic ability, containing at times some foreign bodies taken up in by phagocytosis; and when vital dye-stuff is injected subcutaneously, they store it abundantly. Therefore it is evident that histiocytes show advanced differentiation as an independent cell type in the early stage already.

F. Relation between subcutaneous histiocytes with other similar cells

As was stated before, histiocytes are found in varied figures under different stimulations. Consequently, discrimination of them from other cells in connective tissues, especially in ordinary section preparations, is quite difficult; but it is all the more necessary to elucidate the relationship.

First as to the relation between histiocytes and fibroblasts, it is evidently judged in most cases that the two are of different kind. This is because of the variance in their vital staining figures as well as the cytological findings, which is also verified by the result of embryological study as is mentioned above.

Then as to mast cells, Rohr included them in " Retikulo-histiozytäres System " as he calls it, but the result of the investigation in our Department shows no positive proof for the transition between the two either cytologically or embryologically. Although in H-E staining, they occasionally appear in the form which is often taken for histiocytes because their cell granules flow out. However, metachromasia reaction test reveals the difference without fail.

As to plasma cells, there are nowadays not a few authors who support the assumption of their being reticulum cells or histiocytic origin. As was mentioned before, we often withnessed some cells resembling plasma cells among the cell groups which appeared in inflammatory foci. However, we made certain that the two distinctly show different structure in supravital and May-Giemsa's staining; and also that plasma cells take unique mode of proliferation unrelated to that of histiocytes. So from these results at least, we cannot agree with Rohr, who tried to include this kind of cell in reticulo-endothelial system under discussion.

Next as to the appearance of small round cells in chronic inflammatory foci, they are often called lymphocytic infiltration indiscriminately, but we are convinced cytologically, that such cells are not real lymphocytes but are small histiocytes, as disclosed by the various stimulation experiments on the skin previously described. Thus we were warned against any hasty generalization that the small round cells seen in ordinary section preparation by H-E staining are lymphocytes, and learned that we should always bear in mind the presence of mixed small histiocytes.

As has been stated above, I regard monocytes and histiocytes as two different kinds of cell. Consequently, I cannot agree with some haematologists who call reticulo-endothelial cells especially reticulo-endothelia in circulating blood "Schilling type monocytes."

The theory that epitheloid cells of tubercular nodules and Langhans' giant cells originate in monocytes prevails among both English and American schools of the present day, and in our country also, there are not a few who support this theory; but we have not yet obtained any findings substantiating it as the result of investigations in our Department. In inflammatory foci, blood monocytes appear next to neutrophile leucocytes and do show phagocytic ability to a certain extent, but the result of our investigations showed that they never become larger than 1.5 times the size of neutrophile leucocytes even if they swell to the utmost. From this fact at least we cannot agree with the view that the peritoneal macrophages are identical with monocytes. At the same time we ascertained that monocytes infiltrating into an inflammatory tissues degenerate and deteriorate in a few days, which allows no assumption for their long survival in tissues as in the case of epitheloid cells.

After all, the theory which asserts the monocytic origin of epietheloid cells is only an assumption ignoring various important facts: 1) The reticulo-endothelial cells under stimulation grow round and become isolated showing sometimes compression or lobular nuclei, and 2) The neutral-red vacuoles thereof show rosette formation.

Nature of Reticuloendothelial Cells

From what described above, it is evident that reticulum cells, histiocytes and reticulo-endothelia are three kinds of cell in the said cell system regarding which many questions have been raised.

We could find no basis, up to the present at least, for agreeing with Rohr's theory who tries to group together fibroblasts, plasma cells and mast cells in one category.

Of the three, kinds of cell in the reticulo-endothelial system, reticulum cells and histiocytes are the same in nature. The former form reticular structure with argyrophil fibers, whereas the latter do not because of the absence of argyrophil fibers in the tissue areas where they are. On the other hand, reticulo-endothelia can be distinguished from those two kinds both embryologically and cytologically. So we cannot agree with Kiyono, who designated the latter cells in the isolated state " histiocytes."

Now, the problem concerns the range and nomenclature of the said system. As was mentioned before, we cannot agree with Rohr nor admit Kiyono's view of histiocytic cell system which leaves out the problem of reticulo-endothelia. Also, the claim regarding " Retotheliales System " or " Retikulumzellensystem " is defective in the point that the existence of histiocytes are ignored. Taking histiocytes in to consideration, we prefer " Reticulohistiozytäres System " which include both reticulum cells and histiocytes but in a different sense from Rohr's.

Judging from pathohistological point, it is rather desirable to include all those cells in one system, because the so-called reticulo-endothelia undergo the same type of changes as histiocytes in many cases of pathological processes, in particular, when their functions are highly accelarated. As is the case in Kupffer's stellate cells, reticulo-endothelia become hardly distinguishable from reticulum cells, which fact also supports the above view. From such a view point, we would prefer to adopt the name " Reticulo-endothelial System " which Aschoff put forward and is most widely employed nowadays. In conclusion it is advisable to partially modify prof. Aschoff's classification of the said cell system as below. To add, macrophages in serous cavities have for the first time been classified together with the said cell system as the result of our most recent investigation.

Reticulo-endothelial system

A. Reticulum cells and histiocytes

I) Reticulum cells of pulp cords, follicles, and sinus of lymph nodes
Reticulum cells of lymph follicles.
Reticulum cells of pulp cords and follicles of spleen
Reticulum cells of bone marrow, thymus, lamina propria mucosae of

small intestine

II) Histiocytes of general connective tissues and serous cavities

B. Reticulo-endothelia

Kupffer's stellate cells of live

Sinus-endothelia of bone marrow

A part of capillary endothelia of pituitary anterior lobe and adrenals

(Sinus-endothelia of spleen and lymph nodes belong to a type between this and general proper endothelia).

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Fig. 1

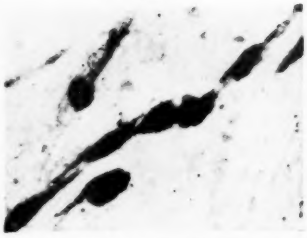

Fig. 2

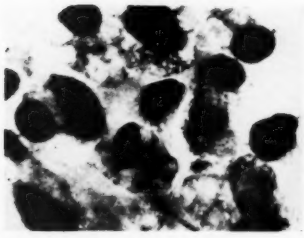

Fig. 1. Strech preparation of subcutaneous connective tissue 1 hour after after auto-blood injection. The subcutaneous histiocytes are found proliferated, minimized in size and arranged in fascicles. (May-Giemsa's stain).

Fig. 2. Ditto. 3 days after auto-blood injection. The proliferated small-sized histiocytes are seen to have swalloned up many erythrocytes, which appear in the form of vacuoles around the nuclei. (May-Giemsa's stain).

Fig. 3

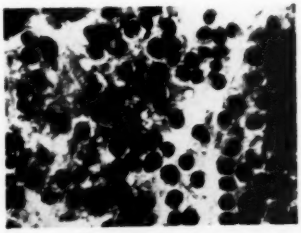

Fig. 4

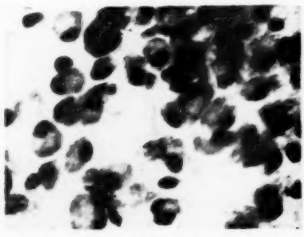

Fig. 3. Strech preparation of subcutaneous tissue of rabbit 5 days after injection with 2% agar solution. Histiocytes reduced in size but enormously proliferated. Cells of such a type are not distinguishable under ordinary staining from lymphocytes, but these cells are highly phagocytic, as those appearing in Fig. 2 above, and are identical with histiocytes in their cytological properties. (May-Giemsa's stain).

Fig. 4. Ditto. 7 days after agar injection. In this stage, the nuclei of the proliferated histiocytes are standing eccentrically, the cells closely resembling plasmocytes. (May-Gimsa's stain).

Fig. 5

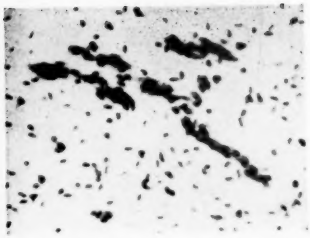

Fig. 6

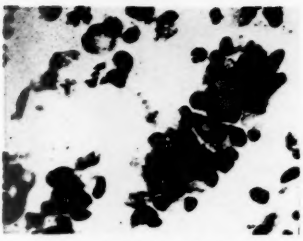

Fig. 5. Strech preparation of subcutaneous tissue following single intraperitoneal inoculation of rabbit with 10 cc. of typhoid fever vaccine. The histiocytes proliferated, showing fascicular arrangement. (May-Giemsa's stain).

Fig. 6. Strech preparation of subcutaneous tissue of rabbit following introduction of fine bone powder. The histiocytes proliferated nest-wise showing the appearance of giant cell. (May-Giemsa's stain).

Fig. 7

Fig. 8

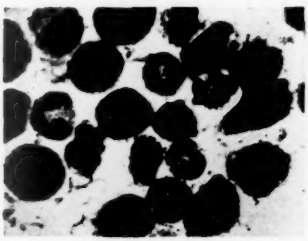

Fig. 7. Markedly proliferated histiocytes adhering to the cover-glass which is inserted in subcutaneous tissue of rabbit 16 days after the intraperitoneal inoculation of 10 cc. of typhoid fever vaccine (12 hours after the cover-glass insertion). (May-Giemsa's stain).

Fig. 8. Findings of cover-glass inserted in subcutaneous tissue of rabbit, which previously intravenous injected with 5 cc. of 1% trypan blue solution every other day for one month (12 hours after the insertion of glass-plate). Many of histiocytes closely resembled monocytes.

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A Case of Congenital Leukemia
The 6th Case Reported in Japan

By

Masayuki Shindo and **Shoji Shibatani**
(進藤 正之) (柴谷 昭治)

From the Department of Pediatrics, Konan Hospital, Kobe.
Pediatrician-in-Chief: Dr. M. Shindo

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Introduction

Leukemia is a relatively rare disease, especially in infancy. As to the congenital or embryonal leukemia, its occurrence is extremely rare as Wintrobe states in his Clinical Hematology[1]: "Leukemia has been observed at birth in about 21 instances ("congenital" leukemia)." Recently we have experienced a case of acute myeloic leukemia, whose blood picture was considerably different from that in the hitherto reported cases of congenital leukemia. Below we shall describe our own case and try to discuss in brief all the 5 cases of the disease hitherto reported in our country.[2)-6)]

Report of Case

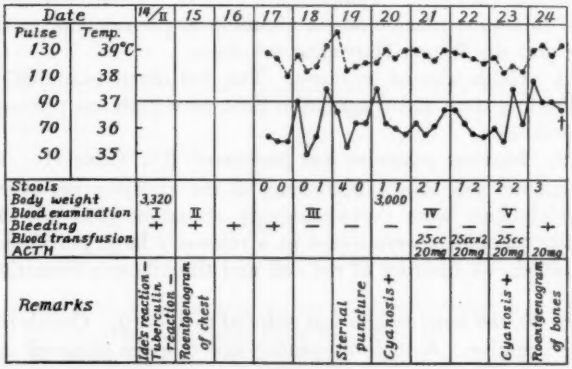

Fig. 1. Course K. O. 22 days (Feb. 14, 1955).

Case: K. O. Girl, 22 days of age
Family; Parents healthy. No miscarriage. Patient was the first child.

No blood disease, no tuberculous or syphilitic disease in the parents.

Past history: Born at full term. 2.700 g. at birth. Breast-fed, though secretion was rather scanty. No malformation.

Present history: born on Jan. 23, 1955. From Feb. 9 on cutaneous and umbilical bleedings occurred, complicated by nasal bleeding on Feb. 13. On advice of the midwife, she was brought to our Outpatient Department.

Clinical findings: body weight, 2720 g. nutrition bad. Face slightly icteric and facial expression apathetic. Nose stuffy. Heart sound pure. No acute rash. Fontanel large. Breath sound equally strong on both sides. White spots on oral mucous membrane and on the tongue. Purpuras, 2–3 cm. in diameter, were seen on the back of the right hand, the left upper arm and the right foot, and pinheadsized petechiae on the abdomen and the right leg. Cervical, axillar and inguinal lymph nodes swollen to the size of soy beans. Abdomen protruded. The liver 4 cm. under the costal margin in the right mamillar line. The spleen 4 cm. under the costal margin in the preaxillar line.

Blood examination showed it to be a case of myeloic leukemia. Peroxidase reaction (of Sato and Sekiya) was negative in 85% of myeloblasts. Tuberculin reaction and Ide's reaction for syphilis were both negative.

Clinical course: The girl visited our Department again on Feb. 15. The site of the puncture of the ear lobe which was performed on the previous day was apt to bleed, and subcutaneous ecchymoses increased in number on the right fore arm. The second blood examination was made (Cf. Table I). Blood type: B. The rentgenogram of the chest showed no abnormity. On Feb. 16, sleep was rather good. Stool twice, of black color. The liver and the spleen were more swollen, the former 8 cm. and the latter 10 cm. below the costal margin. The circumference of the chest was 29 cm.

She was admitted to Hospital on Feb. 17. She was so feeble and delicate. The skin had lost elasticity. Temperature 37.5°C. Treatment consisted in the injections of thiamine, niacine and a vitamine K preparation, further those of 5% glucose and the Ringer-Hartmann solutions.

Feb. 18. Child wanted to nurse. The 3rd blood examination was performed. Bleeding time and coagulation time were both less prolonged. Child cried now and then.

Feb. 19. Marrow puncture was performed (Cf. Table II)

Myelogramm was shown according to the classification of Schleip and Alder.[8] Myeloblasts were overwhelmingly abundant (74.8%). Eosinophiles and especially basophiles were found in a relatively large percentage as in the peripheral blood. A disorder of red cell and thrombocyte formation was easily suspected.

On Feb. 20, the body weight was reduced to 2,500 g. Occult blood of stool had become negative. A spell of cyanosis and dyspnea occurred at night.

On Feb. 21, the 4th blood examination was made. From this day on ACTH injection and blood transfusion were performed.

On Feb. 22, nasal obstipation and disturbed sleep began to occur.

On Feb. 23, the 5th blood examination was performed. Sleep was dis-

TABLE I

Blood Picture of Our Own Case of Congenital Leukemia

Day of examination			14/II	15/II	18/II	21/II	23/II
Hemoglobin (Sahli's value)			142	140	138	118	108
Red cell count (millions)			568	548	524	438	452
Reticulocyte count (‰)			—	6	4	—	4
Normoblasts			10	5	6	5	5
Thrombocyte count			74,200	121,000	189,000	—	109,000
White cell count			99,200	100,100	119,500	88,700	155,300
Leucocytes	Myeloblasts	%	72.5	64.0	65.5	65.0	50.0
		Peroxidase reaction (+)	85	82	83	88	80
		Peroxidase reaction (−)	15 } 100%	18 } 100%	17 } 100%	12 } 100%	20 } 100%
	Promyeloblasts		10.0	5.0	11.0	8.0	10.0
	Neutrophiles	Myeloblasts	1.5	1.0	0.5	0	1.0
		Metamyeloblasts	0.5	1.5	0.5	0.5	1.5
		Stab cells	4.5	6.0	3.5	2.5	9.5
		Segmented cells II	6.0	16.0	10.5	14.5	16.0
		Segmented cells III	5.0	5.5	7.5	9.0	10.5
		Segmented cells IV	0	1.0	1.0	0.5	1.0
		Segmented cells V	0	0	0	0	0.5
	Eosinophiles		2.5	0.5	1.0	0.5	0.5
	Basophiles		2.0	4.0	1.0	5.5	3.5
	Lymphocytes		1.0	1.0	0.5	1.5	1.5
	Plasma cells		0	0	0	0	0
Bleeding time			17′	—	9′ 30″	—	—
Time of coagulation		Begin	35′	—	9′	—	—
		End	38′	—	11′	—	—
Sedimentation of erythrocytes (Yoshida's micro method)		15′	0	0	0.5	0	0.5
		30′	0.5	0.5	1.5	0.5	1.0
		45′	1.0	1.0	2.0	1.0	1.5
		60′	1.5	1.5	2.5	1.5	1.5

turbed due to nasal obstipation and dyspnea. Temperature was 37.8°C.

On Feb. 24, respiration became more frequent.

On Feb. 25, child died at 1.00 p.m. with ever increasing cyanosis.

COMMENT

Congenitality

Tokoro[2] stipulated the occurrence of leukemia in the embryonal age

TABLE II

Bone Marrow Picture

	%	Normal value
Reticulum cells:		
Lymphoid cells	0.3	1.0— 2.0%
Plasma cell like cells	0.3	
Phagocytic cells	0.4	
Unidentified cells	1.0	
Megakaryoblasts	0	0.1— 1.0%
Megakaryocites	1.0	
Myeloblasts	74.8	1.0— 2.0%
Myelocites:		
Neutrophiles immature	3.6	1.0— 4.0%
Neutrophiles mature	1.0	10.0—17.0%
Neutrophiles Meta-	2.0	12.0—18.0%
Eosinophiles immature	0	0.1— 1.0%
Eosinophiles mature	1.0	0.5— 1.7%
Eosinophiles Meta-	0.8	0.4— 1.0%
Basophiles immature	0.3	
Basophiles mature	2.0	
Basophiles Meta-	2.8	
Mature blood cells:		
Neutrophiles	2.9	10.0—15.0%
Eosinophiles	0	1.0— 2.0%
Basophiles	0	0.1— 0.5%
Monocytes	0	1.0— 3.0%
Lymphocytes	1.0	28.0—50.0%
Erythroblasts:		
Megaloblasts	0	2.0—10.0%
Macroblasts	0	
Normoblasts		
polychrom	0.8	
basophil	1.9	
orthochrom	1.0	
Mitotic cells	1.1	

essentially comparable with infantile leukemia and mentioned the following 3 points on the basis of his own experience:

1) An extreme proliferation of blood leucocytes.

2) A limitless proliferation and destructive infiltration into tissues of immature leucocytes resp. atypical ones.

3) The fact that his own case manifested the clinical symptom of leukemia on the 10th day of birth, and the utter impossibility that she could have been born entirely healthy.

Cassilli *et al.*,[9] however, state that a review of literature reveals that the majority of cases reported under this diagnosis indicate there is little or no proof that the disease was actually congenital. . . . Leukemia oc-

curring in the newborn of life is rare. Case reports of leukemia occurring within this age usually call it congenital leukemia.

According to the treatise of O'connor *et al.*[10] recently published the criteria for acceptance as a case of congenital leukemia are not too clearly defined, but the presence of leukemia cells in the peripheral blood, bone marrow, and visceral organs of an infant in the neonatal period is highly suggestive. And they reported a case of congenital leukemia in a 5-day-old boy, with septicemia as a terminal event.

Further, Bernhard *et al.*[11] state: To fulfill the criteria of congenital leukemia, there must be manifest at birth or shortly thereafter, symptoms or signs which can be correlated with characteristic hematologic disturbance. Significant physical manifestations are: spontaneous hemorrhages of skin and mucous membrane, nodular skin infiltrations, enlargement of spleen and liver, adenopathy, fever and pallor. . . . Congenital anomalies were present in 3 of the 4 cases we have reported, indicating a growth disturbance early in fetal life. This opinion of theirs is worthy of attention.

If all these opinions are taken into consideration, occurrence of congenital leukemia is probable, esp. those leukemic cases with symptoms manifested very soon after birth are to be considered with good reason as cases of congenital leukemia. Our own case diagnosed as leukemia on the basis of hematological findings and the diagnose was made on the 22nd day after birth.

Characteristic features of our own case

The main complaint of our own case was multiple purpuras and umbilical hemorrhage, while there was only a slight fever. Hematologically peculiar features were: Firstly, neither red count nor hemoglobin showed a decrease at first; the latter was of a high content, so that no increase of reticulocytes and nucleated reds was seen, nor was the rate of red cell sedimentation accelerated. From Feb. 21 on red cell count and hemoglobin showed a slight reduction though they remained in the lowest figures within the normal range.

Secondly, thrombocytes were 72,000 in number on Feb. 14. Bleeding was prolonged to 17 minutes. The prolongation of coagulation time was more remarkable; blood began to coagulate in 35 minutes and completed coagulation in 38 minutes, so that we had suspected a case of hemophilia before blood morphology was studied. On Feb. 18, however, the prolongation of coagulation time was less pronounced; blood was coagulated within half or one third of the longest prolongation time that the child had ever presented.

Thirdly, white count was about 100 thousand cells, of which 50.0–72.5% were myeloblasts. It is needless to add that hiatus leucaemicus

TABLE III

Synopsis of All Cases of Congenital Leukemia Reported in Japan

Year and author	Sex	Age ; begin, death	Main clinical symptoms	Main hematological findings	Marrow picture	Autopsia	Kind of leukemia	Specific therapy	Remarks
1949 Y. Tokoro[2]	No description	10th day of birth; death after ca 2 months	Stuffy nose ; Liver swelling, 4 fingerbreadths ; spleen swelling, 3 fingerbreadths	Erythrocytes 267 mil. Hemoglobin 48 % Leucocytes 510,000 { Meyloblasts % 3.0; Promyelo. 3.8; Meylocytes 35.4; Metamyelo. 31.6; Stab cells 13.8; Segment 8.4; Lymphocytes 4.3 }	No description	Performed	Myeloic	None	No malformation, no abnormity of bones
1952 K. Takahashi[3] and S. Iizima	♀	Symptoms on delivery; diagnosis on 49th day of birth; death on 59th day of birth	Subcutaneous hemorrhages, pinheadsized petechiae ; liver swelling (2 fingerbreadths) ; spleen swelling (1 fingerbreadth) ; slight fever ; swollen lymph nodes	Erythrocytes 7/11 10/11 Hemoglobin 175 mil. 240 mil. Thrombocytes 34 38 Leucocytes 12,000 24,0000 { Lymphocytes 200,200 184,000 Neutrophiles 97.3% 82.3% Monocytes 0.5% 0.7% 0.2% 0%	No description	Performed	Lymphatic	Vitamine P and Vitamin K, preparation blood transfusion	—
1954 Y. Ito,[4] A. Ton and N. Otashima	♀	Begin with 28th day of birth; diagnosis on 39th day; death on 49th day of birth	Anemia ; dyspnea on feeding ; slight fever ; swollen lymph nodes ; liver swelling (3 fingerbreadths) ; spleen swelling (3 fingerbreadths)	Erythrocytes 27/XII 4/I Hemoglobin 300 mil. 184 mil. Thrombocytes 62 38 Leucocytes 35,000 20,000 { Lymphocytes 810,000 120,000 Neutrophiles 97.5% 99.0% Other kinds 1.5% 0.7% 1.0% 0.3%	Described	Performed	Acute myeloic	None	Incomplete ossification of epiphysis of long bones

955 . Iitsuka[9] nd K. Naoe	♂	Begin with 1 month of birth; diagnosis on 85th day; death on 127th day of birth	Anemia; swollen lymph nodes; liver swelling (2 fingerbreadths); spleen swelling (8 cm.)	Erythrocytes Hemoglobin Leucocytes Myeloblasts Promyelo. Myelocytes Metamyelo. Stab cells Segment Eosinophiles Basophiles Lymphocytes Monocytes	15/VII 209 mil. 50 490,000 48.9% 0 0 0.3 0.5 0.2 0.1 0 0 0	22/VIII 88 mil. 24 19,000 978% 0 0 0.4 0.6 1.0 0 0 0 0	Myeloblasts 97.4% peroxidase reaction+	Performed	Myeloic (myeloblastic)	Urethane 20.5 g. and nitromin 51.0 mg.	Leukemic infiltrations in bilateral testes
955 . Fuchigami,[6] . Hayakawa . Oshige nd J. Arima	♀	Diagnosis on 25th day of birth	Slight anemia; slight fever; liver swelling (3 fingerbreadths)	Erythrocytes Hemoglobin Leucocytes Basophiles Eosinophiles Metamyelocytes Segment Lymphocytes Monocytes		40 mil. 80 13,600 0 % 0 % 2.0% 3.5% 31.0% 61.0%	No description	No description	Monocytic leukemia	None	—
955 . Shindo and Shibatani	♀	Begin with 17th day of birth; diagnosis on 22nd day; death on 33rd day of birth	Subcutaneous and umbilical bleeding; nose stuffy; slight fever, swollen lymph nodes; liver swelling (4 cm.); spleen swelling (4 cm.)	Erythrocytes Hemoglobin Reticulocytes Thrombocytes Leucocytes Myeloblasts	14/II 568 mil. 142 10% 74,000 99,200 72.5%	23/II 452 mil. 108 5% 109,000 155,300 50.0%					

(Cf. TABLE I)

Cf. TABLE II

Not performed

Acute myeloic

ACTH and blood transfusion

No anemia

was seen. The peroxidase reaction of these myeloblasts was negative in 85% of all the cells at the first blood examination, and in 88% at the second blood examination (Feb. 21), though the procent did not amount to 100 even on Feb. 23—2 days prior to death. Further, eosinophiles and basophiles were, though they are rather scanty in usual cases of leukemia, comparatively abundant, the latter amounting to as high a count as 4.0%–3.5% even at the last blood examination. This attitude of myeloblasts, eosinophiles and basophiles is also observed in the myelogram of our own case.

Fourthly, we started the treatment with the vitamines B_1, C and Kativ—a vitamin K preparation (Takeda pharmaceutical Industries Ltd.). On Feb. 21, blood transfusion and ACTH injection were begun, but either clinical symptoms or blood findings did not improve. Dyspnoea and cyanosis became more remarkable and the child died. Though white count had increased to 155,300 cells, the peroxidase reaction of the myeloblasts remained negative in 80% of them.

The Japanese literature concerning "congenital leukemia"

Reports of congenital leukemia are scarce in number too in Japan. The first case (1949) was reported by Tokoro,[2] then the case of Takahashi and Iizima,[3] that of Ito, Kon and Otashima,[4] then that of Iitsuka, Naya and Naoe[5] and further that of Fuchigami, Hayakawa, Oshige and Arima[6] followed. Our own case is thus the 6th case reported of the disease. The synopsis of these cases is given in Table III.

Tokoro's case of embryonal leukoblastosis excepted, our own case was the youngest, as the diagnosis "Congenital leukemia" was made on the 22nd day of birth. Of all the six cases reported, four were of myeloic nature, one of lymphatic nature and the other one was a case of monocytic leukemia; in these cases reported in Japan also myelogenous leukemia was predominant

The course was acute in all the cases and death occurred within one or two months of birth. As to clinical symptoms, the swelling of the liver and the spleen occurred in all the cases. Pallor, hemorrhages and swollen lymph nodes were the next frequent symptoms. It seems that fever was slight as in our own case.

Hematologically, a remarkable decrease of red cells and hemoglobin were observed in all the cases except our own case. The increase of leucocytes were prominent; in all the cases in which platelets were described these decreased remarkably in number. The marrow picture was given in only 3 cases and autopsy findings in 4 cases.

As to "specific" treatments, Iitsuka *et al.*[5] treated their own case with urethane and nitromin, but in the other 4 cases no specific remedy was used. In our own case ACTH and blood transfusion were tried with

no expected effect.

In Iitsuka's case[5] a leukemic infiltration occurred in bilateral tests. This is interesting, if Cassilli's case[9]—a case of acute myeloblastic leukemia in urticaria pigmentosa—is taken into consideration. Further, Bernhard *et al.*[11] state that it seems more than a coincidence that 3 of their 4 cases of congenital leukemia presented multiple developmental anomalies. But in the Japanese literature above tabulated there is no description of any congenital anomaly. Dale[12] writes in his clinical and roentgenographic study of 72 cases that roentgen examination of the skeletal system is a valuable aid in diagnosis. But in the Japanese literature no description of skeletal anomaly was made except for Ito's case[4] in which an incomplete ossification was seen.

Recently Reimann *et al.*[13] have reported a case of congenital leukemia with specific skin nodules. They suggested that skin nodules may be the first sign of congenital leukemia. The skeletal anomalies as well as the skin affection mentioned in the above literature will be noteworthy in cases of congenital leukemia we shall come across in the future.

Summary

1. A case of congenital myelogenous leukemia diagnosed on 22nd day of birth was described. The clinical picture began with subcutaneous and umbilical hemorrhages. Fever remained slight through the whole course.

2. Hematologically there were a number of peculiar features: The case presented no anemia and no acceleration of sedimentation rate. Platelets, however, decreased to a count of 74,000, bleeding time being prolonged to 17 minutes and coagulation time also prolonged considerably. The white count was between 100,000 and 150,000 and myeloblasts were of 72.5% of the count at the commencement. Peroxidase reaction was negative in 80% of the myeloblasts and remained so up to the death. Eosinophiles and basophiles increased in number.

3. Marrow examination showed that myeloblasts occurred in 74.8% of all cells. The other features of the marrow morphology was more or less reflexed in the blood picture

4. All the Japanese cases of congenital leukemia hitherto reported were tabulated, our own case being the 6th one.

5. Our own case was principally treated with ACTH and blood transfusion, but no remission occurred. The patient died with dyspnoea and increasing cyanose on the 33rd day of birth.

Conclusions

A case of congenital leukemia in a 22-day-old infant—the 6th case

of the disease in Japan—is described with several hematological features peculiar to the case.

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Changes in Electric Flicker Values by Vestibular Stimulation

By

Mitsuru Kameyama

(亀山　満)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

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Introduction

Motokawa and Suzuki[1] showed that the difference, ΔS, between the threshold for appearance of electric flicker and that for its disappearance might be used as a measure of fatigue. Since then a number of experiments have been performed by various investigators,[2),4)] in line with the suggestion by Motokawa and Suzuki, and the same method has been applied to detect a slight change in general bodily conditions such as those to be found in the menstrual period,[5)] after X-ray irradiation,[6),7)] and after blood transfusion.[8)] In 1950, Takeyama[9),10)], reported that the value of ΔS increased significantly after rotatory stimulation.

In the present experiment this effect was confirmed, and the dependence of the effect on rotatory stimuli was studied more quantitatively. In addition, it was elucidated that there is a great difference between the effect of stimulation of the semicircular canals and that of the utriculus and the sacculus.

Experimental

Method

The apparatus and the precedure used for measuring electric flicker values, ΔS, were described in a previous paper[11)] so that their description may be omitted here. The apparatus used for rotatory stimulation is shown schematically in Fig. 1. B was a very smoothly rotating board on which a subject was seated. In front of the subject there was an illumination box which was used to keep the visual field of the subject at a constant illumination of about 10 lux. The electrical connection between the stimulating electrodes kept in contact with the subject's head and the electric source of the stimulating apparatus was mediated by mercury contacts, C, so that measurements of ΔS could be carried out not only

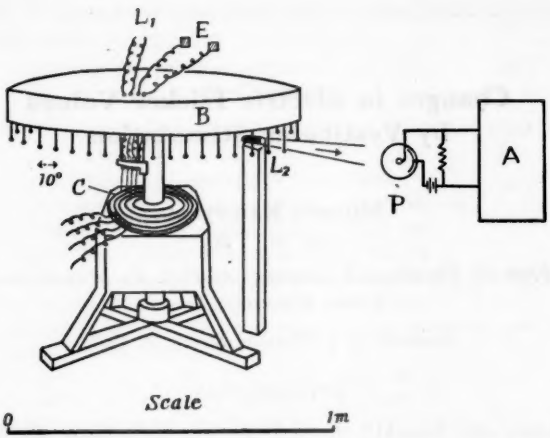

Fig. 1. Apparatus used for rotatory stimulation. A: Amplifier-oscillograph system for photocell, P. B: Rotatory board on which the subject was seated. E: Electrodes for stimulation of the eye. C: Mercury contacts connecting electric sources with stimulating electrodes and electric lamp, L_1. L_1: Illumination box. L_2: Light source for photocell. P: Photocell.

after cessation of rotatory stimulation, but also during it.

The rate of revolution was recorded in the following way; the rim of the revolving board was lined with a series of vertical bars arranged at an equal interval of 10°, and these bars interrupted a beam of light falling into a photocell, P, as the board revolved. The photoelectric current was amplified and recorded by means of an amplifier and an osillograph, A. In Fig. 2 it is shown how constant the revolution of the rotatory board is, except for the beginning and the end of the rotation. As can be seen in this figure, it took about 3 seconds from the start to reach a constant rate in this apparatus, while cessation of rotatory movement was so rapid that the board came to standstill within a second. In consequence, the acceleration was always higher at cessation of movement than at the onset.

The value of flicker, ΔS, was measured immediately after the onset, during, and after cessation of rotation. It took about 30 seconds for obtaining one value of ΔS. Therefore the notation "immedately after" may be understood as "after about 30 seconds."

Results

1) The threshold of rotatory stimulation

The effect of very weak rotatory stimulation upon ΔS values was

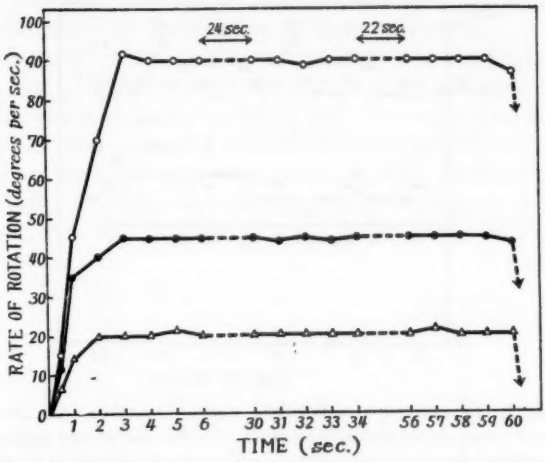

Fig. 2. Graph showing uniformity of speed of the rotatory board. Acceleration occurred only at onset and cessation of rotation.

studied. The position of the head was normal so that the horizontal semicircular canals were to be stimulated.

In Fig. 3 are shown values of ΔS before, during and after a rotatory stimulus at a rate of 5°/sec. (above) and 9°/sec. (below). Each mark represents each value of ΔS. ΔS values were divided into a number of groups, each group containing 5 successive values. These groups were labelled a_0, a_1, a_2—, b_1, b_2—, c_1, c_2 where a, b and c refer to measurements before, during and after stimulation respectively. Taking a_0 as control, the significance of the difference between each group and the control group a_0 was tested statistically, and it was found that the only group which showed a statistically significant difference from a_0 was group c_1 representing values immediately after cessation of the rotation at the rate 9°/sec. No significant rise of ΔS could be observed at the start of rotation. The fact that the off-effect was more effective is probably due to a greater acceleration at " off " than at " on " in our apparatus (see Fig. 2). From this experiment the off-effect of a uniform rotation at a rate of 9°/sec. was the minimal effective stimulus for the horizontal semicircular canals of the subject. The threshold stimuli for the other two subjects were found to be of the same order.

In the following series of experiments the number of revolution was fixed at 6, but the rate of rotation was varied from 18°/sec. to 90°/sec. Values of ΔS were measured successively for 5 minutes before the onset of stimulus, during and immediately after 6 rotations. The results ob-

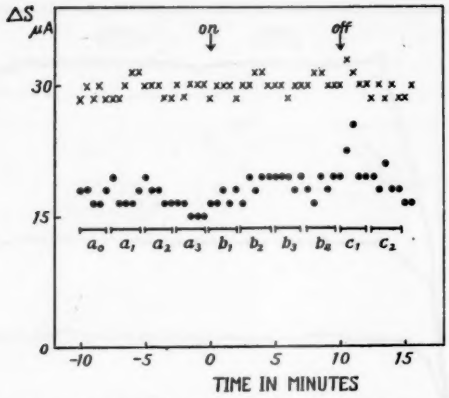

Fig. 3. Determination of threshold rotatory stimulus. All ΔS-values measured before, during and after a rotatory stimulus were plotted against time (data obtained from 2 subjects). Dots and crosses refer to rates of rotation 9° per sec. and 5° per sec. respectively. On: Onset of rotation. Off: Cessation of rotation.

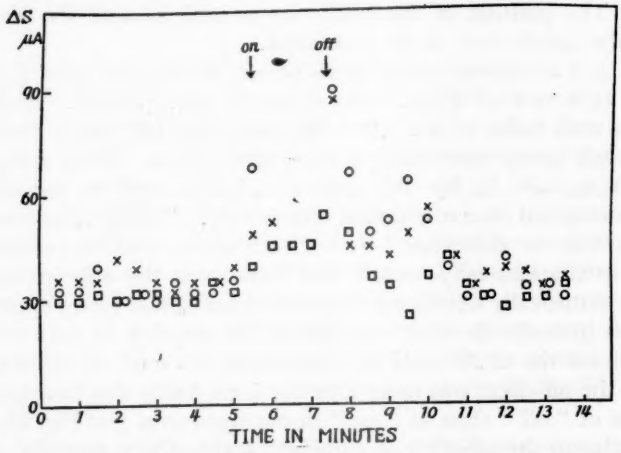

Fig. 4. Dependence of ΔS on speed of rotation. Empty circles, crosses and empty squares refer to speeds of 90°, 45° and 18° per cse. respectively. Data obtained from one and the same subject.

tained are shown in Fig. 4. As can be seen in this figure, ΔS increased immediately after the onset of the stimulus, remained high during the rotation and recovered to the initial level in a few seconds after cessation of the stimulus. When the speed was higher, the value obtained immediate-

ly after the onset or cessation of rotation was especially high. This must be due to the effect of acceleration in these periods.

Next, the effect of the number of rotations was investigated upon ΔS values. In one series of experiment the rate of revolution was fixed at 20°/sec. The result is shown in Fig. 5 (A). In this figure, empty circles, crosses and dots refer to control values before stimulation, stationary values during rotation, and values measured immediately after cessation of rotation respectively. Similar experiments were carried out, fixing the rate of revolution at 30°/sec. and 60°/sec. (see (B) and (C) in Fig. 5).

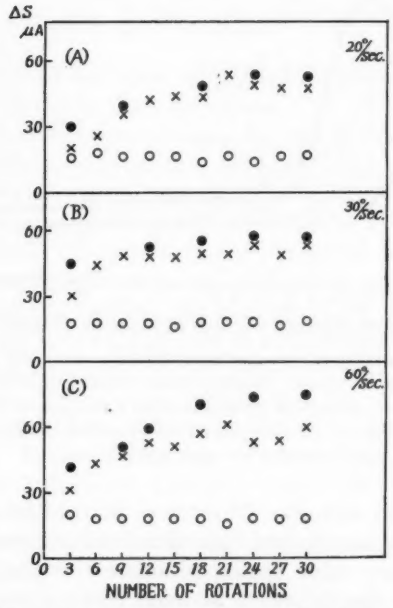

Fig. 5. Effects of number of rotations upon ΔS at three different rates of rotation. In each set of curves empty circles represent ΔS values before stimulation. Crosses and dots refer to values obtained during rotation and immediately after cessation of rotation respectively.

Generally speaking, the value immediately after cessation of rotation is highest, and the difference between this value and the stationary value during stimulation becomes greater as the speed of rotation increases. The effect becomes greater with the number of rotations till about 18 revolutions and further increases in the number of rotations cause no further increase of ΔS.

2) The relation between ΔS and after-nystagmus

For various kinds of constant speed from 18° to 90° per second, the values of ΔS were measured before, during, and after each stimulus of 6 rotations. The duration of after-nystagmus was also determined by inspection as usual in clinical examination. The result is shown in Fig. 6, in which increases of electric flicker values, $\Delta S - \Delta S_0$, caused by stimulation are plotted against the reciprocals of revolving rate or time required

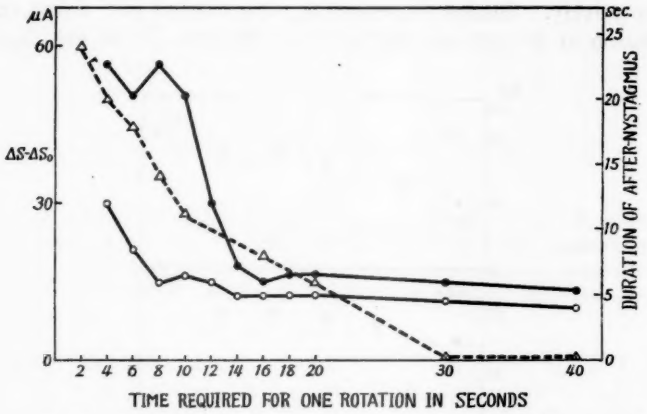

Fig. 6. Duration of after-nystagmus (broken curve) and ΔS measured immediately after a rotatory stimulus (curve marked by dots) and during stimulation (curve marked by open circles) as a function of revolving rate. Ordinates: Increases of ΔS above control and duration of after-nystagmus in sec. Abscissae: Time required for one rotation in sec.

for one revolution in seconds. The curves marked by dots and by open circles refer to the value obtained immediately after cessation of stimulation and to the stationary value during stimulation respectively. As can be seen in this figure, the difference between both curves is not so marked when the revolving rate is relatively low, but as the speed increases above 30°/sec. the difference increases steeply with the rate of rotation. The broken curve marked by triangles represents the duration of after-nystagmus. It declines steadily as the rate is slowed down or the time required for one revolution increases, and becomes zero at a rate of 12°/sec. It is to be noted that increases in electric flicker values and the duration of after-nystagmus do not go parallel in the range of weaker rotatory stimulation. The ΔS value remains raised above the control value, ΔS_0, while no appreciable after-nystagmus takes place, and the difference between ΔS and ΔS_0 for such weak rotatory stimulation is shown to be significant statistically. One may argue that the method of detecting nystagmus

used was not sensitive enough to detect the slightest after-nystagmus which might have occurred after the weakest rotatory stimulation under consideration. In view of such an argument, after-nystagmus was recorded by means of a nystagmograph in some cases, to make comparison with the inspection method. The method of nystagmography has proved to be a little more sensitive than the inspection method; with the former method the duration of the after-nystagmus caused by rotatory stimulation of weak intensity was longer by about 3 seconds than that determined by inspection. Except for the range of too weak rotatory stimulation, there is a parallelism between increases of flicker values, especially those measured immediately after cessation of stimulation and the duration of after-nystagmus.

3) Stimulation of the other semicircular canals

Similar measurements were performed with various positions of the head in space so that different semicircular canals were stimulated. The results obtained were found similar to those obtained in stimulation of the horizontal semicircular canals. In all these experiments the value of ΔS increased above the control value determined before each rotatory stimulation. The magnitude of increase mainly depended upon the magnitude of positive or negative acceleration. When the direction of rotatory movement was reversed rapidly, the increase of ΔS was found especially great, corresponding to a high degree of acceleration thus produced. It is known that most vestibular reflexes show different patterns depending upon the direction of acceleration acting upon the vestibular organ, but no such difference was found concerning the effect upon the phenomenon of electric flicker. From this finding it is likely that the increase of ΔS caused by rotatory stimulation is a sign of some general change following vestibular stimulation.

4) Has statical stimulation of the vestibular organ any effect upon electric flicker values?

It is known that muscular tone of the body and extremities is changed when the position of the head in space is changed (tonic labyrinthine reflex). After the effect of stimulation of the semicircular canals has been studied, it is relevant to make some experiments upon the effect of statical stimulation of the vestibular organ.

The body of the subject was inclined forewards from a horizontal position of lying on the back to various degrees, and in each position measurements of ΔS were carried out. The result is reproduced in Table I, in which positions with the head downwards are marked with stars. The trouble of the subject in such abnormal positions was so great that the time taken for each measurement of ΔS had to be restricted to the necessary minimum, that is, to about 30 seconds. Therefore the subject was brought

to the horizontal position every 30 seconds. It is to be noted that in spite of such great trouble, no change of ΔS was caused by such statical stimulation; ΔS values obtained in the abnormal positions with the head downwards were found little different from those obtained in the horizontally lying position.

In another series of experiments the body was turned backwards from a vertical position, and similar measurements were carried out. The data are represented in Table II. No difference of ΔS could be observed among various positions of the head in agreement with the result of the preceding series.

TABLE I

ΔS Values Measured in Various Positions of the Head in Space

Starred figures refer to positions with the head downwards.

Subjects	Angle in degrees between body axis and horizontal line							
	0	45	90	135	180	225*	270*	315*
	Flicker values ΔS in μA							
M.K.	20.1	19.8	19.5	20.7	20.7	20.4	20.1	20.1
T.H.	26.4	26.4	25.2	25.8	26.1	26.4	25.8	26.4

TABLE II

ΔS Values Measured in Various Positions of the Head in Space

Starred figures refer to positions with head downwards.

Subjects	Angle in degrees between body axis and horizontal line					
	90	67.5	45	22.5	0	-22.5*
	Flicker values, ΔS in μA					
S.K.	18.3	18.6	18.6	18.3	18.3	18.3
J.U.	19.5	19.2	19.5	18.9	18.9	17.4
M.K.	20.4	21.9	21.6	22.5	22.8	20.7

From these experiments it may be concluded that statical stimulation of the vestibular organ causes no change of ΔS whatsoever in contrast with rotatory stimulation.

DISCUSSION

Changes in organs innervated by the autonomic nervous system follow-

ing vestibular stimulation were investigated by Spiegel & Démétriades,[12] by Mies[13] and by others; besides the well known symptomes such as eye nystagmus, vertigo, nausea, vomiting, etc., changes in the cardiac function, pulse rate, blood pressure, secretion and movement of digestive organs were observed. Spiegel emphasized that the sympathetic nervous system is excited by vestibular stimulation.

Takeyama reported that increases in ΔS caused by a rotatory stimulus of a certain definite intensity were significantly greater in patients with inner ear diseases than in normals. Masuda[14] observed that increases of ΔS were caused by an injection of adrenalin in normal adults. But Tarusawa *et al.*[15] reported an opposite effect of adrenalin upon ΔS. Endo[16] could cause a transient fall of both the electric flicker value and the blood pressure by subcutaneous administration of 0.3–0.4 c.c. methobromine or hexamethonium bromide. In any case there is no doubt that the electric flicker value varies with changes in vegetative functions. The striking effect of rotatory stimulation upon ΔS is presumably related to the autonomic disturbance caused by the stimulus.

It is, however, curious that positional stimuli which act upon the utriculus and the sacculus of the vestibular organ had no effect upon ΔS. It is likely, but not yet established that positional stimuli are not so effective as rotatory stimuli in causing autonomic disturbances.

Iwamura[17] and Ikai[18] measured the blood pressure in various positions of the human body and found that the brachial blood pressure depended little on the position, while the blood pressure at the head showed some dependence. These authors are, however, of the opinion that the dependence of the blood pressure at the head on the position is chiefly due to the difference of hydrostatic pressure.

Summary

Electric flicker values or ΔS were measured during and after rotatory stimulation, and it was found that ΔS increases remarkably at the onset and cessation of a rotation.

1. The threshold rotatory stimulus which caused a significant increase in ΔS at a 5 percent level was a rotation at a rate of 9° per second.

2. The duration of after-nystagmus and the value of ΔS measured immediately after cessation of a rotation changed almost in parallel as the speed of rotation was varied, but ΔS was found a little more sensitive to rotatory stimulation than was the after-nystagmus which was measured either by inspection or by an electronystagmograph.

3. ΔS values were measured in various positions of the body in space, and it was found that ΔS depended very little on the position.

From these experiments it was concluded that the electric flicker value

is remarkably influenced by stimulation of the semicircular canals, but very little by stimulation of the utriculus and the sacculus.

I am greatly indebted to Prof. K. Motokawa for his kind guidance and for many valuable discussions.

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Effects of Injection of Analgetics and Alcohol Drinking on Values of Electric Flicker

By

Kiichiro Suzuki, Juzaburo Umetsu, Makoto Kobayashi
(鈴木 亀一郎) (梅津 重三郎) (小林 真)
and **Mitsuru Kameyama**
(亀山 満)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

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Motokawa and Suzuki[1] have previously reported that a flicker sensation of the eye can be used as a very convenient indicator for the measurement of fatigue. Motokawa & Iwama[2] found that the electric threshold for phosphenes was very sensitive to oxygen lack. Suzuki and Umetsu[3] investigated variation of electric flicker value (EF) in response to bleeding and transfusion of blood, and found that an increase and a decrease of oxygen content of the blood cause a decrease and an increase of EF, respectively.

In the present experiment the effects of various analgetics upon EF were investigated.

Experimental

Method

Subjects were 13 healthy men, and 9 of these were injected opium alkaloid solution (2%, 0.5–1.0 cc), 3 were injected benadryl (30 mgr) and phenobarbital (1%, 1.0 cc), and the other was injected thiopental (25%, 12cc).

For about 30 minutes before each injection, the subjects were laid down quietly. A series of measurements of EF and selective reaction time (SRT) were carried out after the rest to obtain control values. Similar measurements were made after the injection at intervals of 10 minutes.

The effect of alcohol drinking was examined on 6 healthy men. 200 cc of 15% alcohol were given to each subject, and the same measurements, as described above, were made.

The apparatus used for the measurements of EF in the medicine injection experiments was the same as Motokawa's original method.[1] In this method the values of EF were expressed in mV. Stimulating elec-

trodes were placed at the outer corner of the eyes of a subject, and the eyes were stimulated with rectangular pulses of 20 cps. The stimulating voltage was increased from zero at a constant rate of 80 mV per second to determine the threshold for the appearance of flicker, S_1, and then the threshold for the disappearance of flicker, S_2, was determined by lowering the voltage from a sufficiently high level at the same rate as above. The EF (ΔS) is represented by the difference of both sorts of threshold. As a value of EF an average of five determinations made in succession was used.

The apparatus used in the alcohol experiments was the same as described recently in Motokawa's report.[4] Sinusoidal alternating currents of 20 cps were applied to the eyes through a pair of silver electrodes. The stimulating current was increased automatically from zero at a constant rate of 12 μA per second, to determine the threshold for appearance of electric flicker, S_1, and then decreased at the same rate to determine the threshold for disappearance, S_2. EF or ΔS was expressed in terms of μA, instead of in mV.

Measurements of SRT were made as follows: One of 3 lamps, red, blue and white in color was lighted in a random order at regular intervals. Subjects switched off the lighted lamp as quickly as possible by tapping the key for this lamp. The reaction time to the red lamp alone was measured, and the value was expressed in msec.

Results

I. Effects of medicine injection

Opium alkaloid injections were made to subjects No. 1 to No. 9. The results obtained are shown in Table I. In each case EF increased gradually after an injection, and reached a maximum about 30 minutes after the injection. Recovery was seen in about 60 minutes. The SRT was measured in cases (subj. Nos. 5, 6 and 9) and indicated in Table I. It can be seen from this table that after the medicine injection the values of EF and SRT run almost completely parallel. It is without saying that the difference between EFs before and after the injections is statistically significant.

On the other hand, we measured the values of EF and SRT after the injection of other kinds of analgetics (benadryl and phenobarbital). The results obtained are shown in Table I, too. These analgetics were found little effective on values of EF and SRT; the difference between values before and after the injection does not seem significant. But the value of EF after a thiopental injection increased remarkably (No. 13 in Table I).

II. Effects of alcohol drinking

TABLE I

Changes of EF (ΔS) and SRT after Injection of Analgetics or other Hypnotics

Subject No.	Injected drug	ΔS in mV and SRT in msec. Time after injection in min.							
			Before	10	20	30	40	50	60
1	Opium alkaloid (2%) 0.6 cc	ΔS	20	172	420	452	400	360	320
2	" 0.6 cc	ΔS	50	160	135	150	180	172	148
3	" 0.6 cc	ΔS	92	150	176	216	208	178	96
4	" 1.0 cc	ΔS	50	188	270	230	384	352	356
5	" 0.6 cc	ΔS	200	400	468	570	570	502	460
		SRT	678	772	820	898	880	842	830
6	" 0.5 cc	ΔS	80	152	172	244	162	186	140
		SRT	497	501	515	660	500	540	540
7	" 0.5 cc	ΔS	88	244	232	132	120	120	110
8	" 0.6 cc	ΔS	136	168	216	140	148	124	130
9	" 0.6 cc	ΔS	36	120	120	200	180	220	228
		SRT	614	607	704	696	707	670	625
10	Benadryl 30mg Papaverin 0.5 cc	ΔS	48	100	52	68	72	140	100
11	Benadryl 30 mg	ΔS	62	124	190	96	90	92	100
		SRT	434	494	510	558	492	498	441
12	Phenobarbital (10%) 1.0 cc	ΔS	200	200	250	220	200	200	200
		SRT	619	622	681	618	628	614	658
13	Thiopental (2.5%) 12.0 cc	ΔS	700	1400	800	850		•	

200 cc of 15% alcohol were administered to each subject.

The results of EF obtained are represented in Fig. 1, A. As can be seen in this figure, the values of EF (ΔS) after alcohol drinking increased gradually and reached a maximum in 30 minutes. 60 minutes after drinking recovery was not yet complete in some subjects. The values of SRT which were measured under one and the same conditions are indicated in Fig. 1, B. It can be seen in this figure that the curves of EF and SRT go almost parallel with each other. Moreover, the effects of the alcohol drinking and the opium alkaloid injection on EF and SRT have remarkable resemblance.

In a subject, 30 minutes after drinking (15% alcohol, 200 cc), a second administration of alcohol (15%, 50 cc) was made. The time courses of EF and SRT were prolonged remarkably, as is illustrated in Fig. 1, C and D.

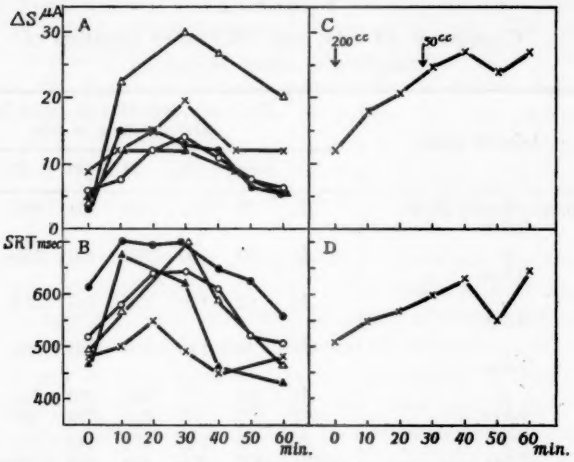

Fig. 1. A and B: Changes of electric flicker values (A) and of selective reaction time (B) after 15% alcohol drinking. C and D: Change of electric flicker (C) and of selective reaction time (D) when a subject drank again alcohol 30 minutes after the first drinking. Abscissas: Time after the first alcohol drinking. Ordinates: Values of EF (ΔS) in μA and SRT in msec.

By means of a photoelectric colorimeter,[6] the alcohol content in the blood of 2 subjects after drinking was measured at intervals of 20 minutes. The EF values in these subjects were measured in parallel. The continuous curves in Fig. 2 refer to the alcohol content, and the broken ones

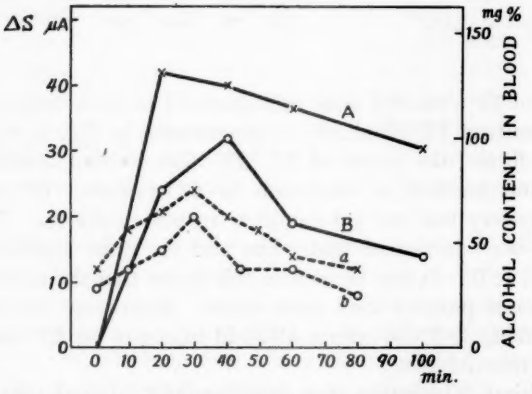

Fig. 2. Variation of alcohol content in blood after alcohol drinking in 2 subjects (A and B). Curves a and b refer to electric flicker values measured in parallel.

to EF values. It can be seen in this figure that the alcohol content and the value of EF increase and decrease almost in parallel.

Discussion

Washburn[5] showed that, when the central nervous system was paralyzed by analgetic injections, values of SRT increased generally.

In our experiment such an increase of SRT always appeared after the analgetic injection (opium alkaloid and thiopental) and after the alcohol drinking as mentioned above. And similar increases were found also in the values of EF. But the injection of benadryl and phenobarbital caused little changes in EF and SRT. It is difficult at present to explain this difference in the mode of action, but it may be supposed that the difference is related to the difference in loci upon which the two groups of analgetics act. Benadryl and phenobarbital are said to act upon the brain stem more strongly than upon the cortex. The other analgetics, especially opium alkaloid, are known as effective narcotics on the cortex.

Summary

By means of Motokawa's method of electric flicker, we measured for 60 minutes the variation of electric flicker values of 9 subjects after opium alkaloid injections, of 3 subjects after analgetic (benadryl and phenobarbital) injection and of 5 subjects after 15% alcohol drinking. In some cases selective reaction time (SRT) was measured in parallel.

1. After the opium alkaloid injections and alcohol drinking, the values of EF and SRT increased gradually, reached a maximum about 30 minutes after an injection or alcohol drinking, and decreased to the initial value in about 60 minutes.

2. In case of benadryl and phenobarbital injections, no significant change of electric flicker values and selective reaction time could be found.

3. In some subjects the alcohol content of the blood was measured together with EF and it was found that both changed almost in parallel.

The authors have pleasure in acknowledging their indebtedness to Prof. K. Motokawa who has guided them kindly.

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Studies of High-molecular Placental Components in Connection with Pregnancy Toxemia

Seventh Paper: On Proteins*

By

Hirosi Yosizaki

(吉崎　宏)

From the Medico-chemical Institute (Director: Prof. H. Masamune) and the Department of Obstetrics and Gynecology (Director: Prof. T. Shinoda), Tohoku University, Sendai

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This series of investigation has been extended to proteins. Five proteins were obtained in electrophoretically homogeneous state from human placentas and investigated chemically and biologically like the non-protein tissue components.[1)-6)] Those proteins showed toxicities to pregnant rabbits, which were not necessarily common to all of them. In conjunction with the findings described in the foregoing communications, it is plausibly concluded that, of the high-molecular placental components, the proteins and nucleic acids are the factors inducing pregnancy-toxemia, whether directly or indirectly, through their combined effects.

Precipitin reaction of the proteins with the sera of non-pregnant and normal pregnant women and pregnancy-toxemia patients and skin reaction of them in those women and patients, puerperal women and uterus-cancer patients were also examined as additional experiments.

Experiments and Comments

Preparation Procedure

Preliminary fractionation

118 fresh placentas were washed, freed from blood vessels and adhering membranes and minced together. The brei which weighed 34.22 kg. was extracted with 1.5 volumes of 0.14 *M* NaCl containing 0.01 *M* sodium citrate through agitation in a freeze mixture for 24 hours. Centrifuged. The centrifugate was further treated in a similar manner two times more, employing decreasing volumes (1.4 and 1.2 volumes) of the mixed salt solution, and the united supernatant fluids (pH 6.8, red turbid) were acidified to pH 4.2 with hydrochloric acid and stood for several hours. The voluminous brown-colored deposit was centrifuged off (Fr. 1). The supernatant was neutralized with

* 12th report of Masamune and co-workers' "On Proteins and Amino Acids."

sodium hydroxide, distilled at 30°C to 1/10 the volume and saturated with ammonium sulfate. The deposit here was filtered off after standing for several hours. It was repeatedly salted out similar to above from about 5 volumes of distilled water, until a Molisch-negative filtrate was given. The final deposit was dissolved in an equal volume of water in a homogenizer and dialyzed for 6 days, when the solution showed a pH of 5.8 and gave a brown precipitate (Fr. 2). Centrifuged. The dark brown supernatant fluid was distilled in vacuo to about 1/10 the volume and acidified to pH 2.0 with hydrochloric acid. The occurring small precipitate (biuret +, Molisch +, Bial +, test for P +, Dische +) was filtered off and rejected, and to the filtrate was added the satd. baryta to pH 9.0. A small precipitate (biuret +, Molisch +, Goldschmiedt +, Bial +, test for P +, Dische +) occurred again, which was also filtered off and discarded. The filtrate here was further treated with hydrochloric acid and baryta as above twice more till no more occurrence of precipitate. It was then liberated from barium by careful addition of dilute sulfuric acid (pH of the solution lowered to 2.3), and the centrifuged supernatant was added to with 10% NaOH to pH 5.0 and dialyzed for 3 days against running water (about 5°C) to eliminate the excessive sulfuric acid. The deposit during dialysis was separated off on a funnel, and the filtrate was cooled to −5°C in a freeze mixture, and after addition of alcohol to 15%, further added to with 10% NaOH to pH 6.0. A brown precipitate occurring was centrifuged off (Fr. 3) and the supernatant fluid (Fr. 4) was subjected to the process in *4*) below.

Isolation of proteins in pure state

1) *Protein I which is insoluble in water at pH 5.0.* Fr. 1 above was biuret ±, Molisch ±, Dische +, Bial-Mejbaum ±, Goldschmiedt + and Neuberg-Saneyoshi ± and contained a marked amount of P, suggesting a mixture of proteins, PNA, DNA and itinsulfuric acids. It was suspended in 50 cc. per placenta of 3 *M* NaCl, added to with 10% NaOH in drops to pH 7.0 under agitation, and after further agitation for 12 hours at 0°C in a freeze mixture, placed in a refrigerator for 40 hours. The turbid solution given was acidified with 10% HCl to pH 4.2. The voluminous brown deposit that occurred was filtered off after standing in the refrigerator for several hours. It was reprecipitated from 3 *M* NaCl with 10% HCl twice as above, added to with 50 cc. per placenta of water and dissolved by means of a homogenizer, dropping in of 10% NaOH to pH 8.0. This solution was dialyzed, a small deposit that occurred in the mean time (probable Protein II) was rejected after centrifugation, and the supernatant was saturated with ammonium sulfate, followed by filtration. The bulk on the funnel was subjected to two reprecipitations, when the filtrate was Molisch-negative, and freed perfectly from ammonium sulfate by 6 days' dialysis. Then the solution was liberated from nucleic acids by shifting the pH to 2.0 with HCl at first and then to 9.0 with baryta, each of the deposits thereby being discarded. After elimination of barium with sulfuric acid (pH of the solution decreased to 2.3), the solution was dialyzed for 2 days until the pH was raised to 5.0. The occurring voluminous deposit here was separated and dissolved in

50 cc. of water by the aid of NaOH. The pH of this solution was lowered carefully with HCl, the precipitates at pH 5.8 and higher being discarded and the precipitate at pH 5.0 being recovered. The substance recovered was dissolved in the same volume as above of water by the aid of HCl, followed by raising the pH with NaOH. Here the precipitates at pH 4.6 and lower were rejected and that at pH 5.0 was recovered again. The fractionated precipitation was repeated ten times in all by shifting the pH from alkaline side five times and from acid side five times. The recovered fraction, which was free from proteins precipitable at other pH values than 5.0, was reprecipitated at pH 5.0 many times until it proved homogeneous electrophoretically. It was finally dissolved in water by adjustment of the pH to 7.0, dialyzed against running water for 3 days and against changes of distilled water for 1 day, concentrated in vacuo and freeze-dried in turn. A yellow-tinged powder was given. Yield 60 mg. per placenta. It was preserved in a vacuum desiccator containing anhydrous calcium chloride.

2) Protein II which is insoluble at pH 5.8. The wet Fr. 2 was stirred up in 50 cc. of water, and 10% NaOH was added till pH 9.0 under continuous agitation. Filtered. The filtrate was repeatedly adjusted to pH 2.0 with HCl and to pH 9.0 with baryta as above, rejecting the precipitates at those pH values (nucleic acids), and subsequently barium was eliminated with 10% H_2SO_4. The solution was then added to with 10% NaOH to pH 5.0 and dialyzed. A voluminous deposit (brownish-colored) settled when the pH of the solution reached 5.8. The precipitate given (biuret ##, Molisch +, test for P −, Goldschmiedt −, Neuberg-Saneyoshi −, Bial ±) was centrifuged off and subjected to 5 repetitions of alternative dissolution in water at pH 8.0 and in water at pH 3.0 together with subsequent shift of the pH with 10% HCl and with 10% NaOH respectively. The small precipitates at pH values other than 5.8 were discarded. The precipitate at pH 5.8 was recovered each time and further reprecipitated at pH 5.8 even thirty times. It was finally dissolved in water by the aid of NaOH and dialyzed, and the solution before getting turbid was distilled in vacuo and freeze-dried. 60 mg. per placenta of a yellowish powder was given. It was kept also in a vacuum desiccator ($CaCl_2$).

3) Protein III which is insoluble at pH 6.0. Fr. 3 (biuret ##, Molisch ++, Goldschmiedt −, Dische −) was dissolved in 30 cc. per placenta of water by addition of 10% NaOH to pH 7.4 and acidified to pH 6.0 with dilute hydrochloric acid. The brownish flocculent precipitate was centrifuged off and dissolved in water by addition of 10% HCl to pH 5.0. pH of the clear centrifuged supernatant was carefully raised with 10% and 1% NaOH to 5.8. And the small precipitate occurring here (Protein II) was discarded and the mother fluid was added to with additional alkali to pH 6.0. A flocculent brownish precipitate appeared after a while. The contaminations precipitable at pH values of 5.8 and lower were removed by repeating the process ten times. Here elimination of Proteins I and II was tried particularly carefully when the pH of the acidic solutions was raised. The fraction given was reprecipitated at pH

6.0 twenty five times and dissolved in 10 cc. per placenta of water at pH 4.0. The solution was dialyzed, condensed and freeze-dried to yield 7 mg. per placenta of a brownish powder. The product was also preserved in vacuo over $CaCl_2$.

4) Protein IV which is precipitable at 2/5 saturation of its solution with ammonium sulfate. Fr. 4 (biuret ##, Molisch ##, Goldschmiedt —, Neuberg-Saneyoshi —, test for P —) was dialyzed, distilled to about 1/5 the volume and after rejecting some precipitate during the dialysis (probable Protein II), 3/10-saturated with ammonium sulfate. After one day, the light brown deposit was filtered off and discarded, and the filtrate was added to with the satd. ammonium sulfate to 2/5 saturation and stood. Filtered. The filtrate (Fr. 5) was set aside in order to separate Protein V (See below). The bulk on the funnel was fractioned in a similar manner three more times employing each time 5 cc. per placenta of water for dissolution, when the filtrate proved Molisch-negative. It was again taken up in the same volume of water, dialyzed for 6 days and distilled in vacuo to about 1/2 the volume. The condensate was warmed to 33°C in an air-thermostat and 6.5/10-saturated with sodium sulfate (21.58 g. of anhydrous sodium sulfate per 100 cc.). The deposit was stood for a few hours and separated on a creased filter in the thermostat. It was submitted to 3 reprecipitations, dialyzed, condensed at a low temperature and freeze-dried. A white powder weighing 20 mg. per placenta was yielded. This substance was also kept in vacuo over anhydrous calcium chloride.

5) Protein V which is precipitable at half saturation of its solution with ammonium sulfate. Fr. 5 set aside in *4)* was added to with the satd. ammonium sulfate to 1/2 saturation, and after standing for 1 day, filtered. The bulk on the funnel was similarly treated four times, employing 5 cc. per placenta of water each time for dissolution, until a Molisch-negative filtrate was given, dialyzed for 6 days after dissolving in the same volume as above of water and filtered. The filtrate was 6.5/10-saturated with sodium sulfate at 33°C to remove Protein IV (The precipitate amounted little) and filtered again. The filtrate here was saturated with the same salt at the same temperature. The occurring deposit was treated three more times in a similar manner and then its watery solution was dialyzed, distilled in vacuo and freeze-dried. A yellowish powder was yielded which amounted to 30 mg. per placenta. It was also preserved in a vacuum desiccator containing anhydrous calcium chloride.

Chemical and Physical Properties of the Proteins (I–V)

Test-tube tests. Their results are embodied in Table I.

Paper partition chromatography of the amino acids in the proteins. a) 5.2–12.1 mg. of substance was hydrolyzed by heating with 6 *N* HCl in a sealed tube at 100°C for 20–24 hours, and the hydrolysate was evaporated in a vacuum-desiccator containing solid caustic soda and anhydrous calcium chloride, adding water from time to time, until the solution showed a pH value higher than 2.0. It was dried up and dissolved in 0.2–0.5 cc. of distilled water, and a 0.02–0.03 cc. portion of the solution was sampled on a 30×30 cm. square of Toyo Roshi

TABLE I

Results of Test-tube Tests of Proteins I–V

Test	Protein I	II	III	IV	V
Biuret	++	++	++	++	++
Molisch	−	−	+	+	+
Goldschmiedt	−	−	−	−	−
Neuberg-Saneyoshi	−	−	−	−	−
Test for phosphorus	−	−	−	−	−
Dische for deoxypentose	−	−	−	−	−
Millon	+	+	+	+	+
Hopkins-Cole	−	−	+	+	+
Sakaguchi	+	+	+	+	+

filter paper No. 2. Primary irrigation was effected with a mixture of butanol, glacial acetic acid and water (4:1:2 by volume) and the secondary with Solvent phenol-water (4:1 by volume) in an ammonia-containing atmosphere. Temperature of test 25°±1°C. Indicator 0.1% ninhydrin in butanol satd. with water.

b) The proteins were hydrolyzed and chromatographed according to Masamune and Hakomori,[7] changing the volume proportion of 0.1 *M* borax and phenol into 7:50 in the solvent mixture for the secondary irrigation.

The chromatograms of the proteins showed in common spots at the positions corresponding to aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, leucine and/or isoleucine, valine and/or methionine, proline, lysine, histidine and arginine. Those of Proteins III–V in a) showed spots of phenylalanine (weak) and of hexosamine in addition.

Quantitative analyses. The analytical figures obtained on the preparations are shown in Table II. It is particularly noteworthy that all of Proteins I–V are extraordinarily rich in proline, because even β-casein, gelatin, elastin and collagen contain no more than 15% proline (These proteins contain 15.1,[9] 14.1,[10] 13.4[11] and 15.1%[9] proline respectively).

Solubility and precipitability of the preparations. The preparations were insoluble in glacial acetic acid, although IV and V dissolved in this solvent when previously kneaded with a small quantity of water. Their watery solutions (pH 5.8–6.5; Some were dissolved in water by the aid of alkali and dialyzed for the examination) precipitated on addition of lead acetate, cupric chloride and trichloroacetic acid and on saturation with ammonium sulfate, but did not on addition of barium-acetate and -chloride.

Electrophoresis. Protein I and II were examined, using their condensed solutions before freeze-drying at the final stage of preparation. The solutions were analyzed as regard to nitrogen content to determine the protein concentrations, dialyzed for 24 hours against a large volume of an alkaline phosphate buffer pH 7.7, and after addition of an appropriate quantity of the buffer, subjected to electrophoresis. Proteins III–V were applied as buffered solutions

TABLE II

Composition of Proteins I–V

Analysis	In per cent					In number of moles per 10^5 g. protein					In molecular ratios, designating tyrosine content 1 unit				
	Prot. I	II	III	IV	V	Prot. I	II	III	IV	V	Prot. I	II	III	IV	V
N*	13.95	15.41	14.35	12.75	13.67										
Amino acids†:															
Aspartic acid	12.18	12.11	12.45	14.77	12.01	92	91	94	111	90	24.03	12.04	10.94	19.52	7.26
Glutamic acid	21.09	14.81	16.17	20.83	18.75	143	101	110	142	128	37.65	13.32	12.85	24.90	10.26
Serine	5.45	5.49	8.46	7.14	1.82	52	52	81	68	17	13.62	6.91	9.41	11.95	1.39
Glycine	3.44	3.56	4.56	2.89	1.68	46	47	61	39	22	12.04	6.27	7.10	6.77	1.80
Threonine	3.71	2.86	3.70	2.56	2.55	31	24	31	22	21	8.18	3.13	3.63	3.78	1.72
Alanine	3.09	3.97	3.00	6.65	6.90	36	45	34	75	78	9.41	5.89	3.94	13.13	6.24
Tyrosine	0.69	1.37	1.55	1.03	2.25	4	8	9	6	12	1.0	1.0	1.0	1.0	1.0
Leucine and/or isoleucine as leucine	7.47	9.88	5.06	7.64	8.15	57	75	39	58	62	14.97	9.97	4.51	10.25	5.00
Valine and/or methionine as valine	2.22	4.75	2.94	3.64	3.40	19	41	25	31	29	4.92	5.35	2.94	5.47	2.34
Proline	18.78	19.24	22.76	16.10	25.05	163	167	198	140	218	42.86	22.12	23.12	24.60	17.52
Hexonebases as arginine	10.50	15.34	7.70	7.72	18.71	55	81	41	40	98	14.50	10.67	4.73	7.14	7.92
Total	88.62	93.38	88.35	90.97	101.27	698	732	723	732	775					
Hexosamine‡	0.0	0.0	1.23	1.80	0.69										
Ash§	4.16	5.45	3.42	4.10	3.17										

*Micro Kjeldahl. †Masamune and Hakomori[7]. ‡Blix.[8] §Pregl without use of H_2SO_4.

of pH 7.7 and of pH 4.6 prepared with the preparations. All the patterns showed one sharp boundary (Fig. 1).

Relative viscosity. All of Proteins I–V were applied without antecedent isolation as a powder. Their solutions dialyzed for 1 day against distilled water at the final stage of preparation (See the preparation procedure) were analyzed as regard to N prior to the viscosity measurement, which was effected in an Ostwald type viscosimeter of Neurath *et al.*[12] Temperature of test 25°C. The results of the measurement are shown in Table III.

Ultraviolet absorption. Another part of the solutions used for measurement of relative viscosity were diluted. The ultraviolet spectra of them are shown in Figs. 2A–2E. Protein V markedly absorbed ultraviolet rays at the wavelengths 275–280 mμ in accord with its comparatively high tyrosine content. Among the other proteins containing less tyrosine, Proteins I and II showed no such absorption which is characteristic to usual proteins (Cf. Table II).

Biological Activities

Most of the biological activities were assayed by Drs. Tanaka, Funakubo, Saito and others and have been partially published.[10]-[15] To the examinations the condensed solutions of the proteins before freeze-drying at the final stage of preparation were applied after addition of NaCl to 0.85%. The results may be briefly described as follows.

1) Histo-pathological findings in liver and kidney when pregnant rabbits were injected with the proteins. 5 mg. per kg. body weight of Proteins I, II, III, IV and V were intravenously (auricular vein) injected into pregnant rabbits as 0.40, 0.29, 0.58, 0.63 and 2.05% solutions in physiological saline respectively. The results are shown in Table IV. Liver lesion occurred, when the proteins, specially Proteins I–III were injected, and particularly noticeable was that Protein I caused anemic necrosis and Protein II the hemorrhagic. On the other hand, Protein IV induced glomerulonephrosis in distinction from the other four proteins. The findings in liver and kidney accord with those in human pregnancy-toxemia.

2) Effect of the proteins on blood pressure. Table V shows the behaviour of blood pressure in carotic artery when urethane-narcotized pregnant rabbits were injected with the proteins in the same dose and manner as in *1)*. Proteins I, III and IV gave rise to noticeable rise of blood pressure in pregnant rabbits, whereas Protein I did not in a non-pregnant rabbit.

3) Effect on urine volume of pregnant rabbits. Pregnant rabbit were deprived of food since the previous night, and after narcotizing, fettered on the back, and the urinary bladder was laid bare, opened and emptied by means of a catheter. Intromission of 100 cc. of luke-warm water into the stomach and injection of Protein I or II in the same dose and manner as in *1)* were processed simultaneously. The urine secreted was collected during the following 4 hours. As shown in Table VI, Protein I proved not to be effective upon the urinary secretion, whereas Protein II definitely decreased the secretion reminding of oliguria in pregnancy toxemia.

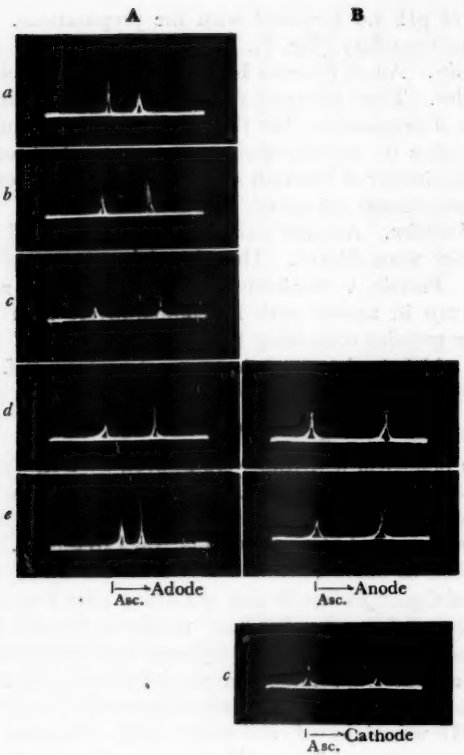


Fig. 1. Electrophoretic patterns (1/2×) of 0.5% solutions of Protein I (*a*), II (*b*) and III (*c*) in phosphate buffer pH 7.7, I 0.2 (**A**) and an 0.5% solution of Protein III (*c*) in acetate buffer pH 4.6, I 0.2 (**B**) and 1% solutions of Protein IV (*d*) and V (*e*) in the phosphate and acetate buffers above. Current 10 mA. Temperature: 7°C in *a***A** and *b***A**; 23°C in *d***A** and *e***A**; 20°C in *d***B** and *e***B**; 17°C in *c***A** and *c***B**. Time of exposure after starting current: 50 min. in *a***A**, *b***A** and *e***A**; 65 min. in *c***A**; 30 min. in *d***A**; 60 min. in *c***B**, *d***B** and *e***B**. Mobility:

$$a\mathbf{A}: \quad \frac{-1.40\times 0.007643\times 0.3}{3000\times 0.01} = -10.70 \text{ cm}^2/\text{volt/sec}\times 10^{-5}$$

$$b\mathbf{A}: \quad \frac{-1.25\times 0.007643\times 0.3}{3000\times 0.01} = -9.55 \quad "$$

$$c\mathbf{A}: \quad \frac{-1.00\times 0.008255\times 0.3}{3900\times 0.01} = -6.35 \quad "$$

$$c\mathbf{B}: \quad \frac{+0.85\times 0.006464\times 0.3}{3600\times 0.01} = +4.57 \quad "$$

$$d\mathbf{A}: \quad \frac{-0.83\times 0.009031\times 0.3}{1800\times 0.01} = -12.49 \quad "$$

$$dB: \quad \frac{-0.10\times0.006310\times0.3}{3600\times0.01}=-0.52 \quad \text{"}$$

$$eA: \quad \frac{-1.48\times0.009031\times0.3}{3000\times0.01}=-13.36 \quad \text{"}$$

$$eB: \quad \frac{-0.08\times0.006310\times0.3}{3600\times0.01}=-0.42 \quad \text{"}$$

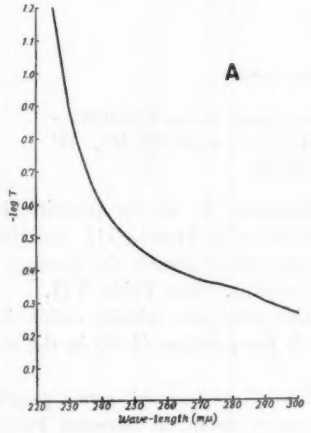
TABLE III

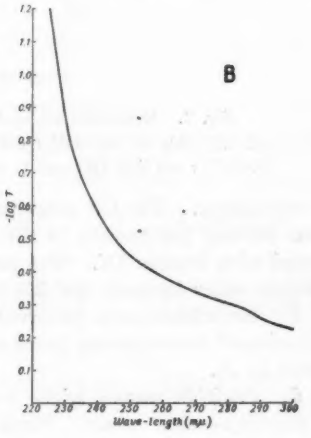
Relative Viscosity of Solutions of Proteins I–V

Temperature of test 25°C. pH of the solutions 6.0.

Protein	Concentration of the solutions (%)	Time of outflow	η_r
I	0.0	56.60	
	0.402	63.56	1.123
II	0.0	61.0	
	0.286	63.0	1.040
III	0.0	56.60	
	0.584	72.08	1.275
IV	0.0	61.0	
	0.803	64.9	1.062
V	0.0	56.60	
	0.675	59.08	1.044

4) Effect of Proteins III–V on concentration of Cl^- in subcutaneous tissue. The chloride ion concentration in subcutaneous tissue was measured potentiometrically according to Takemura[16] in pregnant rabbits before and after intravenous injection of the proteins in the same dose and manner as in *1)*. It was because that, in pregnancy toxemia, the patients become edematous. The rabbits were maintained on a definite food for 10 days prior to submission to





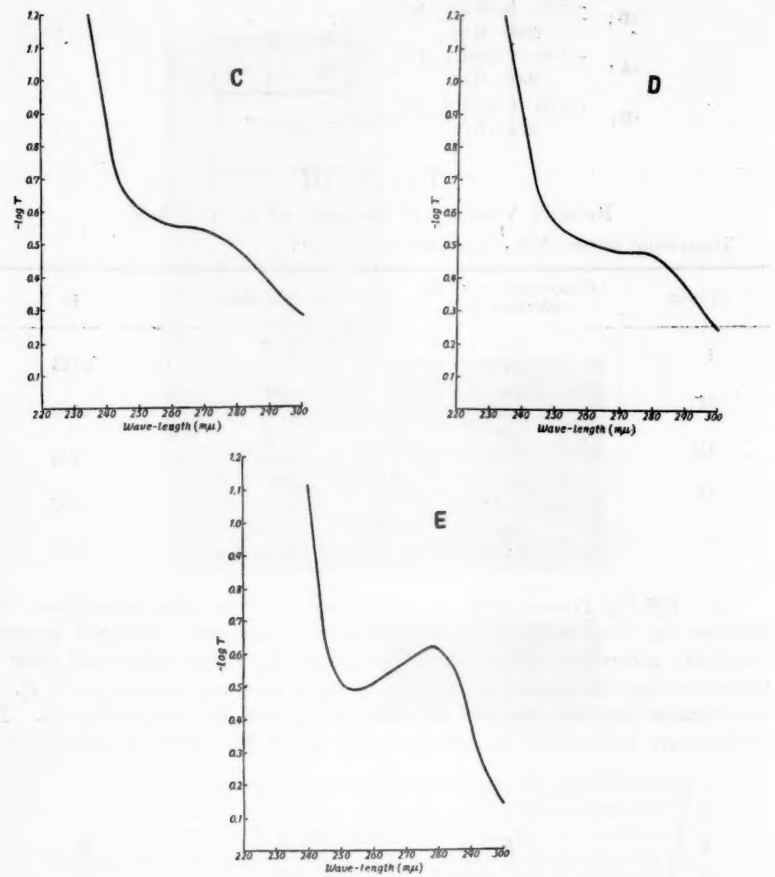


Fig. 2. Ultraviolet absorption spectra of solutions of Protein I (0.0134% ; pH 6.0) (**A**), II (0.0095% ; pH 6.0) (**B**), III (0.02% ; pH 6.0) (**C**), IV (0.031% ; pH 6.0) (**D**) and V (0.01% ; pH 6.0) (**E**).

the experiment.. The Cl^- concentration was increased by all the proteins, but it was striking particularly in the rabbits injected with Protein III and those injected with Protein IV. The proteins thus proved to possess the potency of retaining water beneath the skin in pregnant rabbits. See Table VII.

5) No albuminuria occurred in all of the pregnant rabbits which had been injected intravenously (auricular vein) with the proteins (I–V) in the dose applied in *1)*.

6) Precipitin reaction of the proteins with sera of non-pregnant, normal-pregnant and pregnancy-toxemic women. Weak positive reaction occurred between Protein

TABLE IV

Pathological Changes in Liver and Kidney of Pregnant Rabbits after Intravenous Injection of the Proteins

Regarding the injection dose of the proteins, see the text.

Rabbit No.	Rabbit Wt. (g.)	Days of pregnancy	Time of killing animal after inj. (hrs.)	Liver: Necrosis	Liver: Necrobiosis	Liver: Cloudy swelling	Liver: Degeneration	Liver: Stasis of blood stream	Liver: Thrombosis	Liver: Change of Glisson capsule§	Kidney: Nephrosis	Kidney: Glomerulonephrosis	Premature delivery//	Aggrav. of general status after inj.
Protein I injected														
75	2900	20	48	++‡	+‡	+	++	−	−	+	±	−	+	+++
76	2500	22	46	++‡	−	±	−	−	−	+	−	−	−	++
77	2700	26	42	−	−	−	++	±	−	++	−	−	−	++
78*	3100	24	32*	−	+‡	−	++	−	−	−	−	−	+	+++
Contr. 1†	2650		49	−	−	−	+	−	−	+	−	−		++
Contr. 2†	2500		42	−	+‡	++	−	−	−	+	−	−		+
Protein II injected														
79	2600	22	48	+	+‡	++	±	+	−	+	−	−	+	++
80	3000	23	48	+	+	++	++	−	−	−	−	−	+	++
81	3540	23	48	++	+	++	+	−	+	+	−	−	−	++
82	2900	22	48	+	+	+	+	−	−	−	−	−	−	+++
83	3540	24	48	++	+	+	+	+	−	++	±	−	−	++
84	2760	21	22	++	+	+	++	+	−	+	−	−	+	+++
85	2660	23	45	++	+	+	++	−	+	−	−	−	+	++
Contr. 1†	2800		47	−	±	±	±	−	−	−	±	−		+
Contr. 2†	2340		43	−	−	−	−	−	−	−	−	−		++

TABLE IV—*Concluded*

Rabbit No.	Rabbit Wt. (g.)	Days of pregnancy	Time of killing animal after inj. (hrs.)	Liver: Necrosis	Liver: Necrobiosis	Liver: Cloudy swelling	Liver: Degeneration	Liver: Stasis of blood stream	Liver: Thrombosis	Liver: Change of Glisson capsule§	Kidney: Nephrosis	Kidney: Glomerulonephrosis	Premature delivery//	Aggrav. of general status after inj.
Protein III injected														
86	2800	22	35	−	−	−	−	−	−	−	−	−	−	+
87	2500	26	24	−	−	+	−	−	−	−	−	−	−	+
88	2800	27	24	−	−	−	−	−	−	−	−	−	−	++
89	3500	27	24	−	++	++	+	+	−	−	−	−	−	+
90	3000	23	24	−	+	+	−	−	−	−	−	−	−	+
Protein IV injected														
91	2600	25	24	−	+	+	+	−	−	−	−	±	−	+
92	2500	26	24	+	+	++	+	−	−	+	−	++	−	++
93	2800	25	25	−	−	±	−	−	−	−	−	−	−	+
94	3000	24	24	−	−	±	−	−	−	−	−	±	−	++
95	3000	25	24	−	−	+	−	−	−	−	−	+	−	+
96	3300	26	24	−	−	+	−	−	−	−	−	−	−	++
97	3000	25	24	−	+	++	+	+	−	−	+	+	−	++
Protein V injected														
98	2700	20	20	−	−	+	−	−	−	−	−	−	−	+
99	2200	25	24	−	−	+	−	−	−	−	−	−	−	+

* Died after 32 hrs. from the injection. † A non-pregnant female rabbit was injected. ‡ Anemic. § Cellular infiltration and swelling of the sheath. // + signifies the occurrence.

TABLE V

Blood Pressure in Carotic Artery of Pregnant Rabbits after Injection of the Proteins

Rabbit No.	Rabbit Wt. (g.)	Days of pregnancy	Blood pressure (mm. Hg) Before injection	Time after injection (min.) 0.5	1	3	5	7
Protein I injected								
100	3100	24	82	100	98	**102**	100	96
101	2700	26	120	124	134	138	**140**	**140**
Contr.*	2560		108	110	108	104	104	102
Protein II injected								
102	2900	22	102	94	91	96	95	92
103	3540	24	101	100	104	104	102	103
Contr.*	2800		98	99	100	101	98	
Protein III injected								
104	2800	22	102	96	100	106	104	106
105	2500	26	98	102	110	**116**	108	106
106	2800	27	100	98	108	**112**	**112**	**112**
107	3500	27	102	110	112	**114**	110	112
108	3000	23	108	98	92	110	116	**118**
Protein IV injected								
109	2600	25	102	82	90	98	104	104
110	2500	26	106	104	108	108	**118**	112
111	2800	25	98	92	96	102	102	102
112	3000	24	98	82	90	108	**114**	110
113	3000	25	102	106	110	122	**124**	120
114	3300	26	104	102	108	**112**	110	**112**
115	3000	25	104	104	108	**112**	108	108
Protein V injected								
116	2700	20	106	92	96	112	106	106
117	2200	25	98	86	90	102	100	96

* A non-pregnant female rabbit was injected.

II and one of the sera of 8 normal pregnant women and two of the sera of 7 pregnancy-toxemic women, but faint positive or no reaction between the same protein and the sera of the other women and between the other proteins and sera of non-pregnant, normal-pregnant and pregnancy-toxemic women.

7) The proteins were injected intracutaneously to non-pregnant, normal-pregnant and puerperal women and pregnancy toxemia- and uterus cancer-suffering patients in the same dose and manner as described in a foregoing article[1] of this series of investigation, and the average diameters of the reddened areas were measured after 4 hours also as before. Protein I and III showed tendency to develop larger rednesses in pregnancy toxemia- and cancer-patients than in

TABLE VI

Urine Volumes Secreted by Pregnant Rabbits after Simultaneous Intravenous Injection of Protein I or II and Intragastric Intromission of 100 cc. of Water

Regarding the dose and injection manner of the proteins, see the text.

Rabbit No.	Pregnancy*	Urine volume (cc.) Time after injection (hrs.) ½	1	1½	2	2½	3	3½	4	Total
Protein I injected										
118	+	1.6	1.8	2.9	3.9	4.1	2.6	1.9	2.0	20.8
119	−	1.2	2.5	2.8	3.0	2.2	1.6	1.7	1.5	17.2
Protein II injected										
120	+	2.3	2.2	1.7	1.7	1.2	1.2	0.9	1.0	12.2
121	−	1.6	1.4	2.0	2.8	1.8	1.6	1.0	1.2	13.4
Controls: No protein injected										
Contr. I	+	2.1	2.3	2.5	3.8	2.3	1.8	1.2	1.3	17.3
Contr. II	−	2.8	2.7	3.1	3.5	3.3	1.6	1.2	1.3	19.5

* + and − signify pregnant and non-pregnant rabbits respectively.

TABLE VII

Change of Cl^- Concentration in Subcutaneous Tissue Fluid of Pregnant Rabbits after Intravenous Injection of Proteins III–V

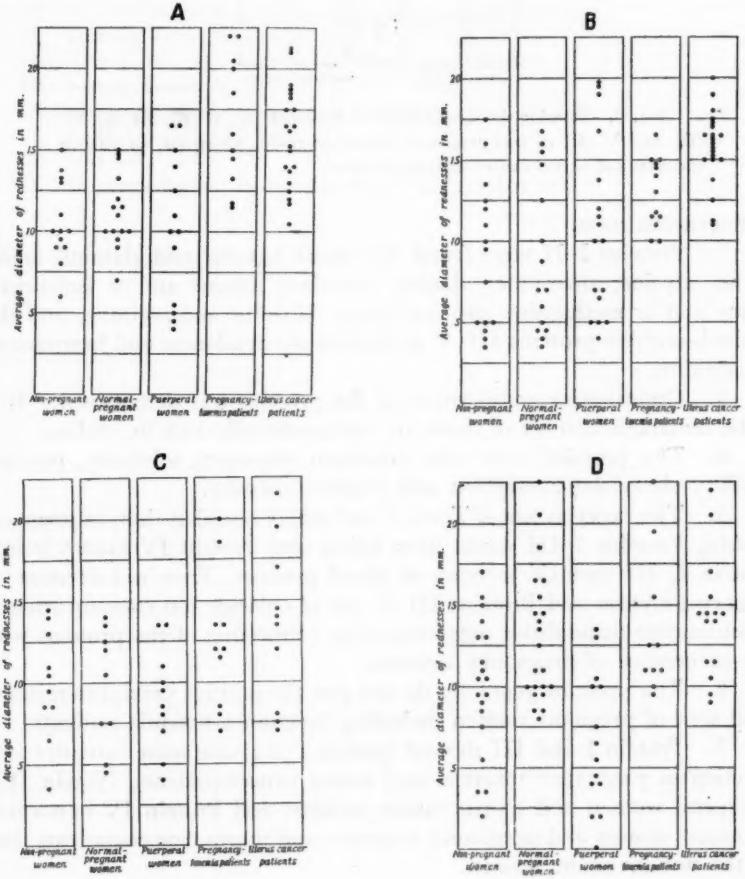
Regarding the dose of the injected proteins, see the text.

Rabbit No.	Wt. (g.)	Days of pregnancy	Concentration of Cl^- in the tissue fluid as NaCl (mM) Before injection	Time after injection (min.) 10	30	60
Protein III injected						
122	2800	22	62	**140**	120	120
123	2500	26	92	110	**130**	**130**
124	2800	27	78	110	**120**	**120**
125	3500	27	75	**125**	110	**130**
126	3000	23	82	112	110	**132**
Protein IV injected						
127	2600	25	65	**110**	75	75
128	2500	26	75	**110**	100	92
129	2800	25	67	**120**	110	88
130	3000	24	65	**120**	100	96
131	3000	25	67	**120**	110	92
132	3300	26	67	**120**	92	92
133	3000	25	67	**120**	100	96
Protein V injected						
134	2700	20	62	**92**	78	78
135	2200	25	68	**88**	**88**	**88**

non-pregnant women, Protein II that of inducing larger rednesses in puerperal and cancer-suffering women than in the non-pregnant, and Protein IV that of developing larger rednesses in normal-pregnant, pregnancy toxemia- and cancer-suffering women than in the non-pregnant. See Figs. 3A–3E.

Summary

1. A protein insoluble at pH 5.0 (Protein I), a protein insoluble at pH 5.8 (Protein II), a protein insoluble at pH 6.0 (Protein III), a protein precipitable at 2/5 saturation with ammonium sulfate of its solution (Protein IV) and a protein precipitable at 1/2 saturation with ammonium sulfate of its solution (Protein V) were separated in electrophoretically



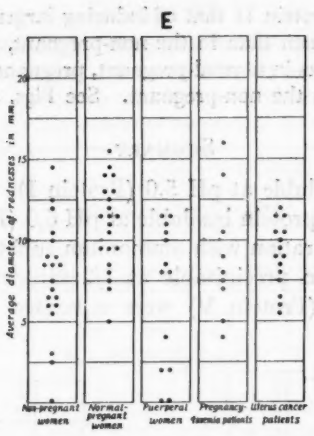


Fig. 3. Showing the skin reaction of Protein I (**A**), II (**B**), III (**C**), IV (**D**) and V (**E**) in non-pregnant, normal-pregnant, puerperal, pregnancy toxemia- and uterus cancer-suffering women.

homogeneous state.

2. Proteins I–II were found to contain aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, leucine and/or isoleucine, valine and/or methionine, proline, lysine, histidine and arginine, and the Molisch-positive proteins III–V to contain phenylalanine and hexosamine in addition.

3. Quantitative composition of the proteins was determined. It is to be remarked that all of them are extraordinarily rich in proline.

4. The proteins were also examined regarding solubility, precipitability, ultraviolet absorption and relative viscosity.

5. The experiments of Tanaka and others revealed that, in pregnant rabbits, Proteins I–III cause liver lesion and Protein IV kidney lesion, Protein I, III and IV increase of blood pressure, Protein I decrease of urinary secretion and Proteins III–V rise of chloride ion concentration in subcutaneous tissue fluid, suggesting close connection of the proteins with the occurrence of pregnancy toxemia.

6. The proteins generally do not give the positive precipitin reaction with sera of pregnant women including pregnancy-toxemia patients.

7. Protein I and III showed tendency of giving more intensive skin reaction in pregnancy toxemia- and uterus cancer-patients, Protein II in puerperal women and uterus cancer patients and Protein IV in normal-pregnant women and pregnancy toxemia- and uterus cancer-patients than in the non-pregnant women.

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Biochemical Studies on Carbohydrates

CCVI. Mucopolysaccharides and Mucoproteins in Pig Lung
Second Paper: Hexuronic Acid-containing Mucopolysaccharides

By

Katuhiko Tokita
(鴇田 克彦)

From the Medico-chemical Institute (Director: Prof. H. Masamune) and Prof. T. Nakamura's Clinic of Internal Medicine, Tohoku University, Sendai

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In 1949, Utusi[1] published papers on an anticoagulative fraction of pig lung which was a probable mixture of hexuronic acid-containing mucopolysaccharides. By a mildest way of fractionation, the writer has established that pig lung parenchyma contains ordinary heparin, most probable β-heparin and chondroitinsulfuric acid as the components in question. The fractionation procedure consisted in 1) extraction of the tissue, previously exhausted with physiological saline and 10% $CaCl_2$, with 10% Na_2SO_4, 2) deproteinization with phenol and a cationic surface active agent and by Sevag technique and 3) fractionated precipitation with ethanol in the presence of barium acetate.

Experiments and Comments

Preparation Procedure

1) Material and treatments with physiological saline and 10% *$CaCl_2$*

Fresh pig lungs were freed from adhering membranes and adipose tissues, trachea, bronchi and blood vessels mechanically as far as possible, washed with water and minced. 1.15 kg. of the brei thus given was shaken with 2 volumes of 0.14 *M* NaCl for 1 day under cooling on ice. The red-brown extract was set aside after centrifugation, and the tissue residue was again treated in a similar manner. The extracts were separately dialyzed in cellophane bags against running water, distilled in vacuo to syrupy consistency and precipitated with 1 *l.* of abs. ethanol. The deposits were washed with ethanol and dried in a desiccator to yield red-brown powders amounting to 68 and 25 g. The products united were Goldschmiedt- and Neuberg-Saneyoshi-negative.

The tissue residue after the second extraction was washed with water (1 *l.*) and shaken under cooling as above with two 2.3 *l.* portions of 10% $CaCl_2$, each extraction taking 1 day. The yellow-brown extracts here were treated as

follows. Each extract was dialyzed for 2 days, condensed to 400 cc. and added to with 40 g. of anhydrous sodium sulfate, followed by standing for 4 hours. The flocculent deposit was centrifuged off and washed with a sodium sulfate solution of the same concentration as the mother fluid. The supernatant fluid and washing were warmed together to 35°C, saturated with sodium sulfate and filtered with suction, maintaining at that temperature throughout. The bulk on the funnel was washed with 100 cc. of the satd. sodium sulfate at the same temperature. The filtrate and washing were diluted three times with water, dialyzed against running water for 2 days and after distilling in vacuo to 100 cc., again dialyzed for 2 days. The fluid was further condensed to a syrupy solution (50 cc.), and 10 volumes of abs. ethanol were added. The fibrous precipitate was washed with two changes of abs. ethanol and dried. Yielded were 1.5 and 0.7 g. The products mixed together proved Molisch ++, Goldschmiedt ±, Neuberg-Saneyoshi −, biuret ± and Dische ++ and contained P (++). The substance thus looked to be chiefly DNA. Because of the positive Goldschmiedt reaction, though faint, it was dissolved in water, precipitated with benzalkonium chloride, separated from proteins with ethanol and subjected to fractionation by the aid of lead acetate according to Homan and Lens.[2] No fractions showing marked Goldschmiedt reaction were given. For caution's sake, the fraction corresponding to heparin was dissolved and added to with $BaCl_2$ and glacial acetic acid in turn after Kuizenga and Spaulding.[3] The deposit, however, was found to be nothing but DNA contrary to expectation (It was biuret −, Molisch −, Goldschmiedt −, Bial +, Dische ++, Neuberg-Saneyoshi −, Elson-Morgan −). The mother fluid was distilled in vacuo to dryness and also tested, but either of Goldschmiedt and Elson-Morgan tests resulted only in very weak positive.

2) *Extraction with 10% sodium sulfate*

New 1.05 and 1.3 kg. batches of the pig lung brei were treated with physiological saline and 10% $CaCl_2$ as above. And all the tissue residues including the one above were united, washed with distilled water and agitated in 3 *l.* of 10% (w/v) sodium sulfate. The mixture was then adjusted to pH 7.0 with 10% NaOH and shaken for 2 days at room temperature (16–18°C), followed by centrifugation. The yellowish, brown, somewhat turbid extract was set aside. The centrifugate was again treated with 2 *l.* of 10% sodium sulfate in a similar manner. The first extract was added to with some Celite and filtered with suction. The yellowish, almost clear filtrate was distilled in vacuo to 500 cc., whereby it turned opaque due to precipitation of proteins and crystalline sodium sulfate. It was placed in an air-thermostat at 35°C for 1 hour and after addition of some Celite, filtered under suction in the thermostat. The bulk on the funnel was washed with 100 and 50 cc. portions of the satd. sodium sulfate at the same temperature. The clear, yellow filtrate and washings were diluted three times, dialyzed for 2 days, and after condensation to 500 cc., again dialyzed for 2 days. It was further condensed to a syrup, precipitated with 300 cc. of abs. ethanol, washed with 200 cc. of abs. ethanol twice and dried in a desiccator. 11.7 g. of a yellow powder was given. The second extract gave a cor-

responding substance weighing 6.2 g. which was mixed together with the former powder. Molisch ++, biuret ++, Goldschmiedt ++, Neuberg-Saneyoshi +.

3) Treatment with 90% phenol

The product was kneaded with less than 1 cc. of water, and 200 cc. of 90% (w/w) phenol was added under agitation. The centrifuged supernatant (transparent and yellowish brown-colored) was discarded, and the centrifugate was further treated with 100 cc. of 90% phenol twice in a similar manner, washed with abs. ethanol containing some anhydrous sodium acetate thoroughly and dried. 4.8 g. of a grey powder was yielded. It still gave positive biuret (+) and Molisch (++) reactions. Goldschmiedt ++, Neuberg-Saneyoshi +.

4) Fractionation by means of a cationic surface active agent (Cf. Scott[4])

It was taken up in 200 cc. of water, the small insoluble part was centrifuged off and discarded, and the solution was adjusted to pH 7.0 with glacial acetic acid and added to with 40 cc. of 10% (w/v) benzalkonium chloride in water, whereby a flocculent precipitate occurred. After standing for 1 hour, the deposit was centrifuged off and washed with two 100 cc. portions of 1% benzalkonium chloride. (The supernatant fluid was Molisch ++ but Goldschmiedt −.) It was then exhausted with 200, 200 and 100 cc. portions of abs. ethanol. The extracts were combined, added to with 8 g. of anhydrous sodium acetate and placed in a refrigerator overnight. Centrifuged. The uncolored, transparent supernatant (Molisch −, Goldschmiedt −) was rejected. The centrifugate was washed with two 200 cc. portions of a mixture of abs. ethanol and abs. ethanol saturated with anhydrous sodium acetate in the volume proportion of 3:1 and with two 200 cc. portions of abs. ethanol and dried. 990 mg. of a powder with silver luster was given. Test for P −, Molisch ±, Goldschmiedt ++, biuret ±. Its watery solution absorbed light neither at the wave-lengths near 270 mμ nor at those near 260 mμ, indicating absence of proteins containing cyclic amino acids, nucleic acids and benzalkonium ions.

In order to completely eliminate proteins, it was submitted to Sevag technique.

5) Deproteinization according to Sevag

It was dissolved in 50 cc. of water containing 7.5 g. of sodium chloride (pH of the solution 7.0) and shaken with seven 100 cc. portions of a chloroform-amylalcohol mixture in the volume proportion of 4:1 in succession, when no gel layer appeared on centrifugation of the mixture. The centrifuged upper layer which was transparent and light yellow, was dialyzed against running water overnight at first and then against distilled water for 1 day, distilled in vacuo to a syrup and precipitated with a mixture of 100 cc. of plain abs. ethanol and 10 cc. of abs. ethanol saturated with anhydrous sodium acetate. Followingly, it was washed with changes of abs. ethanol, adding some anhydrous sodium acetate when necessary, and dried. 630 mg. of a white powder was given. Biuret −, Molisch ±, Goldschmiedt ++.

6) Fractionation with ethanol in the presence of barium acetate

It was dissolved in 20 cc. of water (pH of the solution 7.2), stood in an air-thermostat at 30°C and added to with 5 cc. of 25% (w/v) barium acetate.

The mixture was gradually cooled and stood in a refrigerator (0°C) for 2 days. The white deposit was centrifuged off, washed with 25 cc. of 5% barium acetate previously chilled and abs. ethanol and placed in a desiccator to yield 85 mg. of a non-colored powder—Fr. I. The supernatant and the washing by means of 5% barium acetate were united (50 cc. in total), 5 cc. (1/10 the volume) of abs. ethanol was added, and the strongly turbid mixture was placed in the refrigerator overnight, followed by centrifugation. The centrifugate was washed with abs. ethanol and dried. 245 mg. of a white powder was given—Fr. II. The ethanol concentration of the supernatant was increased to 50% (It is assumed that the volume contraction did not occur when the supernatant and ethanol were mixed together), stood in the refrigerator and centrifuged. The centrifugate was washed with abs. ethanol and dried as above—Fr. III. Yield 370 mg. The supernatant here was added to with abs. ethanol to 75%, and after standing at 0°C overnight, centrifuged. The centrifugate was also washed and dried. 33 mg. of a yellowish powder was given—Fr. IV. The substance recovered from the mother fluid was neither Molisch- nor Goldschmiedt-positive. The Molisch and Goldschmiedt tests of the fractions resulted as follows:

Fr.	Molisch	Goldschmiedt
I	−	++
II	−	++
III	+	++
IV	+	±

Namely, Fr. IV was found to be almost free from hexuronic acid-containing mucopolysaccharide.

Of the markedly Goldschmiedt-positive fractions, Fr. I was changed into sodium salt:—

It was dissolved in 20 cc. of water at 35°C, 1 cc. of the satd. sodium carbonate at room temperature was added, and the mixture was stood at 0°C for 1 hour and centrifuged. The centrifugate was washed with two 5 cc. portions of 1% sodium carbonate, and the supernatant and washings were united, acidified to pH 4.6 and precipitated with three volumes of abs. ethanol. The deposit was washed with abs. ethanol, dissolved in 5 cc. of distilled water and adjusted to pH 7.0 with 0.1 *N* NaOH free from the carbonate. The solution was precipitated with 40 cc. of an ethanol-ether mixture in the volume proportion of 1:1, and the deposit was centrifuged off, washed with 40 cc. of ether and dried. 57 mg. of a white powder was given—Prep. I. It proved homogeneous electrophoretically (See below).

Fr. II was at first treated like Fr. I for electrophoretical examination. Its solution buffered at pH 4.6 showed two moving boundaries besides the stationary. Therefore, 190 mg. of the mixed sodium salts was again fractioned in the manner above. To describe in some detail, it was taken up in 20 cc. of water, added to with 5 cc. of 25% barium acetate and chilled. The small deposit was removed and the clear mother fluid was precipitated with ethanol at the same ethanol concentration and temperature as when Fr. II itself was prepared. The deposit (barium salt) here was changed back into sodium salt—Prep. II.

Yield 90 mg.

Fr. III was taken up in 25 cc. of 5% barium acetate and subjected to ethanolic fractionation at 0°C. The very small deposit at the ethanol concentration of 9% was rejected and the deposit at the ethanol concentration of 30% (The residue after distilling the mother fluid was Molisch + and Goldschmiedt ±) was washed with abs. ethanol and dried. The substance given which was Molisch ± and Goldschmiedt ++, was changed into sodium salt. 250 mg. of a white powder was obtained—Prep. III.

Properties of the Preparations

Electrophoresis. The preparations were examined in both acidic and alkaline buffers. All showed one stationary and one migrating boundary within at least 1 hour after starting current (Fig. 1). To ascertain that the stationary boundaries owed to salt effect, Prep. III was freed and again examined as follows. 30 mg. of Prep. III was dissolved in 10 cc. of water and passed through a column

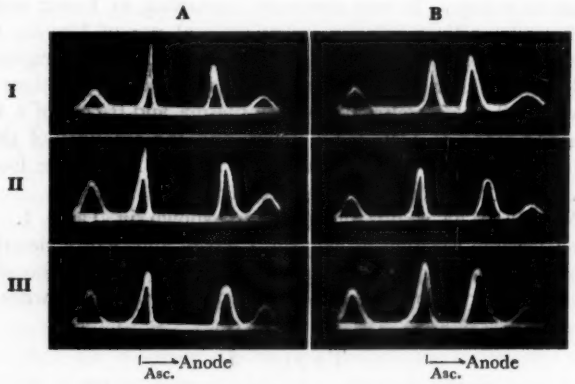

Fig. 1. Electrophoretic patterns (2/3×) of 1% solutions of Prep. I (**I**), II (**II**) and III (**III**) in phosphate buffer pH 7.7, I 0.2 (**A**) and acetate buffer pH 4.6, I 0.2 (**B**). Temperature 10°C; current 8 mA. Exposure 30 min. after starting current. Mobility:

Prep. I at pH 7.7 (**IA**): $\frac{(-1.05)\times 0.007586\times 0.3}{0.008\times 1800} = -1.66$ cm²·volt⁻¹·sec⁻¹ × 10⁻⁴

at pH 4.6 (**IB**): $\frac{(-1.30)\times 0.005505\times 0.3}{0.008\times 1800} = -1.49$ "

Prep. II at pH 7.7 (**IIA**): $\frac{(-0.95)\times 0.007586\times 0.3}{0.008\times 1800} = -1.50$ "

at pH 4.6 (**IIB**): $\frac{(-1.05)\times 0.005505\times 0.3}{0.008\times 1800} = -1.20$ "

Prep. III at pH 7.7 (**IIIA**): $\frac{(-1.0)\times 0.007586\times 0.3}{0.008\times 1800} = -1.58$ "

at pH 4.6 (**IIIB**): $\frac{(-1.15)\times 0.005505\times 0.3}{0.008\times 1800} = -1.32$ "

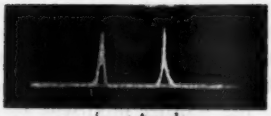

Fig. 2. Prep. III was turned into free acid and subjected to electrophoresis in acetate buffer pH 4.6, I 0.2 (See the text). Temperature of test 10°C; current 8 mA. Exp. 30 min. after starting current. Mobility: $\frac{(-1.10)\times 0.005505\times 0.3}{0.008\times 1800} = -1.26\ \mathrm{cm^2 \cdot volt^{-1} \cdot sec^{-1}} \times 10^{-4}$. 2/3×

(0.8×10 cm.) of Dowex 50 (H^+ form). The effluent and washing were distilled in vacuo at below 30°C, and the still-residue was dissolved in 3 cc. of the same acetate buffer as that used above to submit to electrophoresis. The pattern (Fig. 2) showed only one moving boundary.

Anticoagulant activity. It was measured according to Foster and Nutley,[5] employing heparin sodium salt for investigational use of Upjohn Co. as the standard heparin. Namely, 0.8 cc. portions of the solutions, original and progressively diluted, of the standard heparin and of the preparations were each added to with 1.0 cc. of a citrated bovine plasma and 0.2 cc. of a $CaCl_2$ solution in turn and stood at 37°C for 3 hours, and the amounts of the preparations effecting "50% clot" were calculated. Preps. I–III were found to contain 137, 1.1 and <0.5 U.S.P. units/mg respectively.

Test-tube tests. The results of assays are shown in Table I. Regarding the naphthoresorcinol reaction of heparin, Charles and Scott[6] described that it gives no positive reaction at all and Jorpes and Bergström[7] found a strongly positive reaction on one occasion only. However, the writer's Prep. I

TABLE I

Test-tube Tests of Preps. I, II and III

0.3% solutions of the preparations were applied to the tests excepting Neuberg and Saneyoshi's.

Test	Prep. I	II	III
Biuret	−	−	−
Molisch	−	−	−
Goldschmiedt	++	++	++
Neuberg-Saneyoshi	++	++	++
Osaki-Turumi			
Direct	−	−	−
Indirect	−	−	+
Bial-Mejbaum for pentose	−	−	−
Dische for deoxypentose	−	−	−
Test for phosphorus	−	−	−

which is considerably anticoagulative, showed always the markedly positive reaction.

Paper partition chromatography. a) Hexuronic acid. 10 mg. of substance was sealed with 1 cc. of 1 *N* H_2SO_4 in a tube and hydrolyzed by heating at 100°C for 15 hours. When cold, the hydrolysate was neutralized with the satd. $Ba(OH)_2$ to pH 6.0 and centrifuged, and the centrifugate was washed with three 2 cc. portions of hot water. The supernatant and washings were combined and passed through a 0.9×1.6 cm. column of Dowex 50 (H^+ form) to eliminate barium and hexosamine, and the effluent (pH 3.2) and washing were distilled in vacuo at below 30°C to dryness and dissolved in 0.05 cc. of water. Three 0.01 cc. portions of this solution were sampled separately on slivers of Toyo Roshi filter paper No. 3. One of the slivers was irrigated in a descending way with a mixture of butanol, pyridine and water (5:3:2 by volume), a second with a mixture of butanol, ethanol and water (4:1:5 by volume) and a third with a mixture of *n*-butylacetate, acetic acid, butanol, methanol and water (3:2:2:1:1 by volume). Indicator the *p*-anisidine-phosphate reagent of Mukherjee and Srivostava.[8] The chromatograms of the hydrolysate of Prep. III gave a spot corresponding to glucuronic acid or spots corresponding to glucuronic acid and glucurone, whereas, in the case of Prep. II, that developed with the first solvent showed a spot corresponding to L-iduronic acid* and those developed with the other solvents two spots slightly preceding L-iduronic acid and L-idurone severally. See Fig. 3. The hydrolysate of Prep. I, whose chromatograms are omitted in Fig. 3, gave no spot at all.

b) Hexosamine. In the cases of Preps. II and III, the hexosamine which was absorbed by the column of Dowex 50 above was eluted with 5 cc. of 1 *N* HCl, and the eluate was evaporated in vacuo over solid caustic soda repeatedly, adding water from time to time, and dried up. The residue was dissolved in 0.05 cc. of water (The pH of the solution 5.6) to apply 0.01 cc. of the solution to chromatography. In the case of Prep. I, 5 mg. of it was hydrolyzed with 1 cc. of 4 *N* HCl (Cf. Wolfrom *et al.*[9]) in a sealed tube by heating at 100°C for 12 hours. The hydrolysate was freed from the hydrochloric acid as above, and the evaporated residue was dissolved in 0.05 cc. of water. A 0.02 cc. portion of the solution was applied to chromatography.

Paper slivers Toyo Roshi filter paper No. 3. Irrigation descending. Solvent: the mixture of butanol, pyridine and a dilute HCl solution (5:3:2 by volume, pH 5.5–6.0) of Masamune and Yosizawa[10]. Indicator the aniline hydrogen phthalate reagent of Partridge. As shown in Fig. 4, Prep. I gave a spot corresponding to glucosamine and Preps. II and III that corresponding to galactosamine.

Quantitative analyses. The analytical figures obtained are embodied in Table II.

Optical rotation in water.

		On dry basis
Prep. I:	$[\alpha]_D^{\text{t}} = \frac{0.540 \times 1}{0.0098 \times 1} = +55.1°$ (pH 6.8)	+59.3°

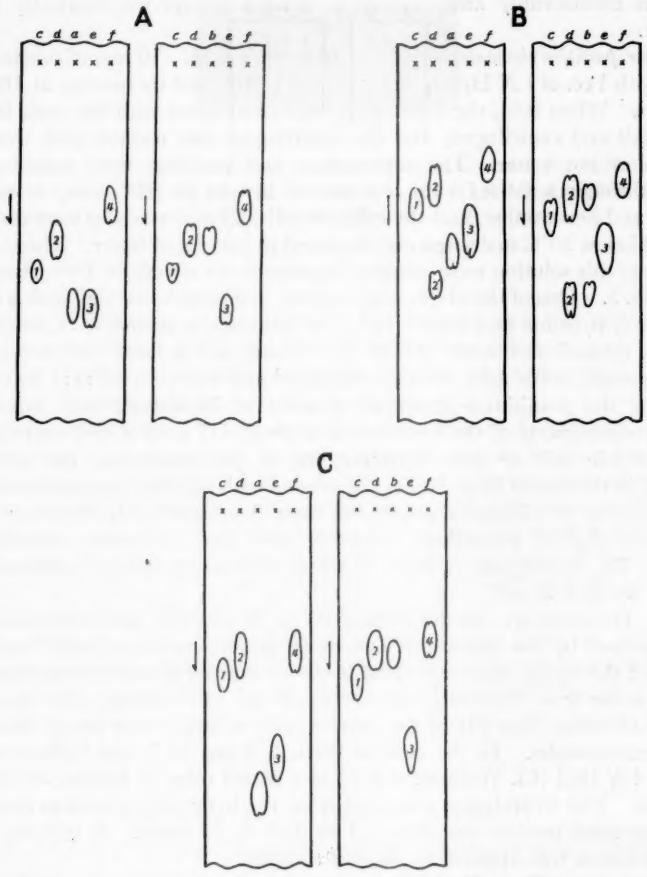

Fig. 3. Hexuronic acid paper chromatograms (1/7×) of the hydrolysates of the preparations. Irrigated with Solvent butanol-pyridine-water at 23°C for 30 hrs. (**A**), with Solvent butanol-ethanol-water at 25°C for 18 hrs. (**B**) or with Solvent *n*-butylacetate-acetic acid-butanol-methanol-water at 23°C for 12 hrs. (**C**). Reference runs effected with 0.01 cc. of 2% solutions of hexuronic acids. The chromatograms of the hydrolysate of Prep. I are omitted.
a Prep. II ; *b* Prep. III ; *c, d, e, f* reference run ; *1* mannuronic acid ; *2* glucuronic acid ; *2'* glucurone ; *3* L-iduronic acid ; *4* galacturonic acid.

Prep. II : $[\alpha]_D^{\alpha}=\frac{-0.479\times 1}{0.0094\times 1}=-51.0°$ (pH 6.8) $\quad -55.7°$

Prep. III : $[\alpha]_D^{\alpha}=\frac{-0.294\times 1}{0.0105\times 1}=-28.0°$ (pH 6.4) $\quad -30.3°$

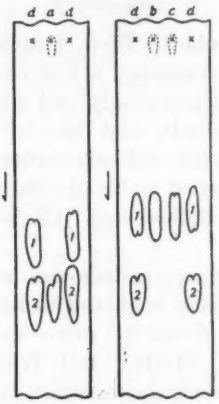

Fig. 4. Hexosamine paper chromatograms (1/7×) of the hydrolysates of Preps. I, II and III. Irrigated with the mixture of butanol, pyridine and a dilute hydrochloric acid (See the text) at 23°C for 36 hrs. Reference runs effected with 0.015 cc. portions of a 2% solution with respect to individual hexosamine hydrochlorides.

a Prep. I; *b* Prep. II; *c* Prep. III; *d* reference run; *1* galactosamine; *2* glucosamine.

TABLE II

Analyses of Preps. I, II and III

Analysis	In per cent			In equivalents per equivalent weight		
	Prep. I	II	III	I	II	III
N*	2.2	2.7	2.9	1.3	1.2	1.3
Hexosamine†	21.8	28.4	28.9	1.0	1.0	1.0
Hexuronic acid as glucuronic acid						
Mannose-thioglycolic acid method‡	8.4	19.9	30.7	0.4	0.6	1.0
Carbazole method§	41.6	24.3	31.8	1.8	0.8	1.0
Acetyl//	1.3	6.5	7.6		1.0	1.1
Sulfur¶	11.0	5.6	4.5	2.8	1.1	0.9
Ash**	33.4	23.4	19.9			
Na††	10.8	7.6	6.4	3.9	2.1	1.7
Moisture‡‡	6.7	8.9	7.2			

* Micro Kjeldahl. † Masamune and Yosizawa[21]; Hydrolysis was carried out by 12 hours' heating with 4 *N* HCl at 100°C. ‡ Dische[22]. § Dische[12]. // Suzuki.[13] ¶ Sakamoto[23]. ** Pregl. †† Calcd. from sulfated ash as sodium sulfate. ‡‡ The substances were dried to a constant weight by heating in vacuo at 110°C over $CaCl_2$ for 3–5 hrs.

Discussion

The strongly anticoagulative Prep. I must be ordinary heparin, because it is made up of glucosamine, a hexuronic acid, which reacts with mannose-thioglycolic acid less strongly and with carbazole more strongly than glucuronic acid as Dische and Meyer[11] and Dische[12] mentioned already, and sulfuric acid in the molecular ratios of 1 : 1 : 3 and is remarkably dextrorotatory. It is regarded to be devoid of acetyl, since the acetyl value obtained is within analytical error (Cf. Suzuki[13] and also Masamune *et al.*[14]).

Prep. II is composed of acetylgalactosamine, a hexuronic acid identical with or resembling L-iduronic acid and sulfuric acid in molecular proportions of 1 : 1 : 1 and is distinctly more levorotatory than chondroitinsulfuric acid. In 1951, Marbet and Winterstein[15] discovered "β-heparin" made up of acetylgalactosamine, a hexuronic acid, which is asserted to be L-iduronic acid by Cifonell *et al.*,[16] and sulfuric acid in equimolecular proportions and has $[\alpha]_D$ of $-60°$. Therefore, Prep. II is most probably identical with β-heparin. It scarcely prevented coagulation, when assayed by Foster and Nutley method. This, however, is explainable on Yamashina's finding[17] that the anticoagulant potency of β-heparin can be demonstrated by the thrombin method[18] but not by other in vitro and in vivo methods.

Prep. III is constituted of galactosamine, glucuronic acid, sulfuric acid and acetic acid in equimolecular proportions and has the specific rotation of $-28°$ nearly in accord with chondroitinsulfuric acid preparations of Masamune and else.[19),20] It may have been partially derived from bronchi remaining uneradicated from the lung parenchyma, which was used as the material for preparation.

Summary

From fresh pig lungs (parenchyma) were isolated three hexuronic acid-containing mucopolysaccharides ordinary heparin, most probable β-heparin and chondroitinsulfuric acid.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

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* We are greatly indebted to Prof. M. L. Wolfrom for 1,2-*O*-isopropylidene-L-iduronic acid.

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An Experimental Study on Transplantation of the Aortic Arch*

Part. I. An Experimental Study on Interruption of the Ascending Aorta

By

Takao Takahashi
(高橋 孝雄)

From the Department of Surgery, Tohoku University Faculty of Medicine, Sendai. Director: Prof. S-T. Katsura

(Received for publication, April 22, 1957)

Introduction

Remarkable advances have been made in cardiovascular surgery in recent years. Especially, in the field of vascular surgery, not only the peripheral blood vessels and abdominal aorta, the thoracic aorta is the subject of surgery. The latter which once considered to be difficult to interrupt for a long period under normal body temperature is now possible to interrupt its circulation by applying hypothermia and various shunts. Restoration of the vascular continuity after resection of the abdominal aneurysm has also become feasible. In case aneurysm involves the ascending aorta and aortic arch, and when malignant tumor in the chest extends to this region, the surgical approach, however, has been considered to be difficult. Since Hardin *et al.*[1] in 1952 reported an experimental study on transplantation of the aortic arch, sporadic clinical cases on this subject have appeared in the literatures. As one of the application of severe hypothermia which is studied by Watanabe *et al.*[2] at this clinic, the author attempted to transplant the aortic arch after the cessation of circulation for a long time under hypothermia without aid of shunts. As a preliminary experiment, cessation and reanastomosis following division of the ascending aorta under various rectal temperatures were performed to study its applicability.

Experimental

Method

Anesthesia to obtain severe hypothermia

* Read in part at the 9th Annual Meeting of the Japan Thoracic Society, Tokyo, October 22, 1956.

After subcutaneous injection with 0.5–0.8 cc. atropine, the dog was anesthesized with injection of Ravonal 15–20 mg./kg. body weight. Then, endotracheal tube was inserted, which was connected to a closed circuit anesthesia machine. When the 3rd phase of 3rd plane anesthesia was obtained, the whole body including the head was immersed into a ice water tub in a supine position. In case shivering was noticed during cooling, ether was added to the machine till it stopped. 100% oxygen was used for respiration. Though the dogs were allowed to breath by its own will, assisted respiration was started when it became weak at rectal temperature 20°–23°C and controlled respiration after it completely stopped. Rectal temperature was all kept below 20°C (19.6°–14°C). When ECG showed cardiac arrest, the animal was immediately removed from the ice water, wipped dry and operation started. The time for cooling was 50–140 minutes.

Operation

The chest was opened at the left 4th intercostal space without cutting the sternum. The ascending aorta was freed from its origin till branching of the right brachiocephalic artery distally. In case complete dissection was impossible, a longitudinal incision was placed in the pericardium and the fat tissue at the reflecting point of the pericardium was excised.

Interruption of the circulation to and from the heart

The ascending aorta was interrupted with a Satinsky's clamp covered with rubber which was placed just above the aortic valve in 15 of 19 dogs. 4 were interrupted under severe hypothermia with simultaneous interruption of the intrapericardial superior and inferior vena cavae by constricting the Nelaton's tube and the ascending aorta just as before. Release of the interruption of inflow tract and the outflow tract in the order given was carried out.

Division of the ascending aorta and its reanastomosis

After the cessation of blood flow of the heart, a Satinsky's rubber shod clamp was placed on the right brachiocephalic artery adjacent to the point where it came off the aortic arch and the ascending aorta was divided in between. 10% sodium citrate solution was frequently injected into the inside to prevent blood clot formation. The posterior row was first anastomosed by twisting 2 supporting stitches on both corners 180 degrees, which was followed with anterior anastomosis. The sutures used were black silk No. 3. Anastomosis was placed intimately with an interval 1–1.5 mm.

Cardiac resuscitation and rewarming

After the interruption was released, the clamp on the central side was removed and the heart was massaged several times, which was followed by removing the distal placed clamp. The dog was transferred from the

operating table to a tub filled with warm water kept at about 40°C. With its left side upwards, it was placed on a vinyl cloth, which was covered over the water. Placing the dog in a slight Trendelenburg's position, cardiac massage was instituted, while the dog was hyperventilated via artificial intermittent respiration during warming process. Cardiac massage was effected by placing both palms of hands intrapericardially, with a rate of 24–30 times a minute, which was increased to 60 times a minute in accordance to the elevation of body temperature. Starting cardiac massage, cardiac standstill was converted to ventricular fibrillation or rhythmic contraction of the right atrium within 4–5 minutes. Counter shock was, however, not used till the rectal temperature rose over 28°C, when 0.2–0.8 cc. noradrenaline was injected into the left ventricle. Counter shock was applied to the fibrillating heart which became active by injection of drug. After defibrillating the heart, in case the cardiac rhythm was weak, 2% calcium chloride 10–15 cc. was injected slowly through the sublingual vein. Confirming that the cardiac contraction became strong, the dog was removed from the tub and chest closed with a draining tube inserted. Rewarming was continued till the rectal temperature returned to normal. The total time required for warming the body was 100–140 minutes.

Results

Group with interruption of the ascending aorta

1) Under normal body temperature. All 3 dogs died by massive bleeding at the clamped site of the ascending aorta within 2–4 minutes. Cardiac standstill and ventricular fibrillation were, however, not observed.

2) Under hypothermia (rectal temperature 28°–24.5°C). 4 dogs were used, of which 3 died from rupture at the clamped site 11, 14 and 18 minutes, respectively. Macroscopically, all cases but one didn't show arrhythmia. Another was interrupted for 19 minutes at rectal temperature 14.5°C. 3 minutes after the release of clamps, bradycardia and arrhythmia occurred, which was followed with ventricular fibrillation. Cardiac massage and warming were both started immediately. 0.6 cc. noradrnaline was injected into thel eft ventricle at rectal temperature 28°C and counter shock applied, which brought temporary normal rhythm to the heart. Eventually the dog died an hour later.

3) Under severe hypothermia (rectal temperature 17°C, 14.5°C) (Table I). In dog No. 24 cardiac resuscitation was successful. The dog died, however, an hour later with central respiratory paralysis. The circulation of No. 25 was interrupted for 70 minutes at rectal temperature

TABLE I

Cessation of the Ascending Aorta at Severe Hypothermia

Dog No.	Body weight (kg)	Rect. temp. (°C)	Time of interruption (min.)	Cardiac resuscitation	Survival time	Cause of death
24	8.0	17.0	34	Success	one hour	Paralysis of respiration
25	6.5	14.5	70	Success	one year	Living

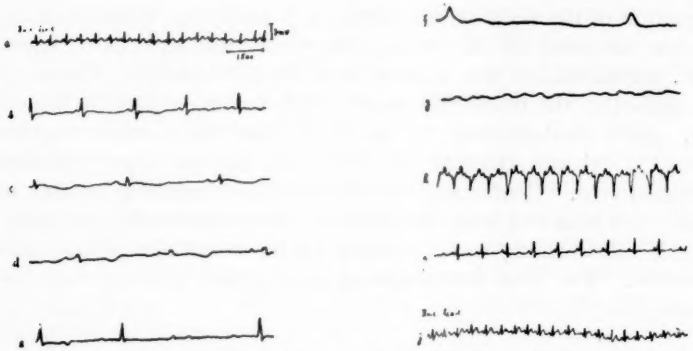

Fig. 1. Electrocardiographic tracing in dog No. 25. *a*: body temperature 36°C. (control ECG, 2nd lead). *b*: rectal temperature 22°C (during cooling). *c*: 16.5°C (during cooling). *d*: 14.5°C (during cooling). *e*: 25 minutes after interruption of blood flow. *f*: 50 minutes after interruption of blood flow. *g*: 70 minutes after interruption of blood flow (release of clamp). *h*: right after counter shock (heart out of pericardium). *i*: rectal temperature 33°C (during rewarming). *j*: 8 days after operation.

14.5°C, and it is still living one year later. No postoperative sequelae has been observed. The ECG are reproduced in Fig. 1. With fall of rectal temperature, tendency of sinus bradycardia is observed. At 22°C, negative T, notching of ST, prolongation of PR interval and QRS; at 14.5°C, wide P, remarkable decrease of ST, reversed T, notching in QRS were observed. 15 minutes after cessation of blood flow, prolongation of P, and amplitudes of QRS were noticed. Extrasystoles were observed 25 minutes later, complete A-V block 50 minutes later, and transition to ventricular fibrillation with release of obstruction 70 minutes later. After successful defibrillation after rewarming to rectal temperature 28°C, tachycardia and elevation of QRS were noticed, and return to normal tracing was noticed on the 8th postoperative day.

Group with division and reanastomosis of the ascending aorta under severe hypothermia (*rectal temperature* 18°–12°C)

1) Interruption of the ascending aorta alone (Table II). 6 dogs were used. No permanent survival was obtained. The time for anastomosis was 20–57 minutes at rectal temperature 18°–14.5°C, while the time

TABLE II

Division and Reanastomosis of the Ascending Aorta under Severe Hypothermia

Dog No.	Body weight (kg)	Rect. temp. (°C)	Time of interruption (min.)	Cardiac resuscitation	Survival time (hrs.)	Cause of death
21	12	16	32	Success	2	Bleeding from anastomosis.
22	13	18	48	Success	0	Bleeding from clamped site.
23	8	17	30	Failure		Mishappening of injecting citrate.
26	14	13	38	Success	0	Bleeding from anastomosis.
27	12	15	65	Success	2	"
28	14	15	60	Success	1	"

for cessation of blood flow was 32–65 minutes. All cases but No. 23 were successfully resuscitated. The cause of death in Nos. 21, 26, 27 and 28 is due to rupture at the anastomosis. Bleeding was not observed during cardiac massage when blood pressure was low, whereas massive bleeding occurred when normal heart beat returned after electric shock was applied. Rupture at the clamped site was the cause of death in No. 22, which occurred by twisting the clamp during posterior anastomosis. The injury was overlooked, and the death was not due to overburden on the heart (Fig. 2).

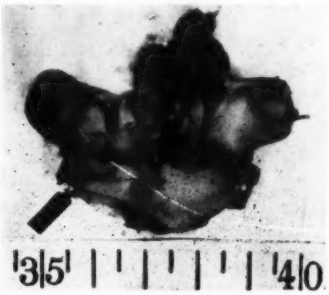

Fig. 2. Injury of intima at the left lower corner (↑) is observed in dog No. 22.

2) Cessation of inflow and outflow tract of the heart (Table III). 4 dogs were used. The time for interruption was 49–75 minutes. All but No. 31 were resuscitated, but no permanent survival was obtained. The cause of death was obstruction due to thrombus at the descending thoracic aorta and the ascending aorta, respectively. Bilateral pneumothorax occurred in No. 34 during operation, which showed atelectasis of lung at autopsy (Fig. 3).

TABLE III

Anastomosis of the Ascending Aorta with Simultaneous Cessation of the Inflow and Outflow Tract of Heart

Dog No.	Body weight (kg)	Rect. temp. (°C)	Time of interruption (min.)	Cardiac resuscitation	Survival time (hrs.)	Cause of death
31	15.0	15.0	65	Failure		
32	11.5	13.0	75	Success	4	Shock due to obstruction of the descending thoracic aorta.
33	11.0	12.0	49	Success	4	Thrombus at anastomosis of the ascending aorta.
34	9.0	14.0	53	Success	48	Collapse of lung.

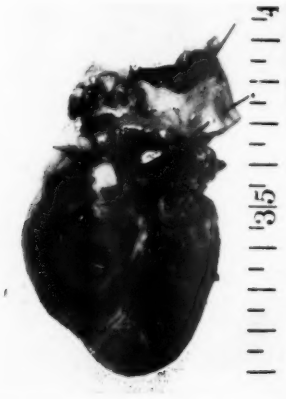

Fig. 3. Removed specimen in dog No. 34.

DISCUSSION

When the ascending aorta is interrupted, the blood flow to the brain is completely shutt off. Cessation of cerebral circulation using such method is reported only by Nakayama[3] and Cooley *et al.*[4] The dog in their experiments died in cardiac arrest which occurred after a few heart beats

or 4 minutes later in an over distended ventricle. The author experienced that rupture at the clamped site occurred within 2–4 minutes before cardiac arrest. Kimoto *et al.*[5] observed that in case the inflow tract of the heart and the aorta were both obstructed at the same time over 5 minutes, all dogs died in cardiac arrest or ventricular fibrillation with loss of cerebral function after 3 minutes. The survival limit of interrupting cerebral blood flow depends on the condition which it was carried out. It is believed that 3 minutes is the limit at normal body temperature.

After advances in hypothermia, it has become possible to prolong the cessation of blood flow of the heart. Marshall *et al.*[6] found out that when dog was cooled to 26°C, the tissue oxygen consumption would diminish about 1/3. They hypothesized that it would be possible to prolong the cessation of circulation 3 times longer in a dog at this temperature, which was substantiated by performing various method of cessation. In our hands, as the dog died from rupture at the clamped site, nothing can be said about damage of cerebral functions. According to Toyoshima[7] at this clinic, the oxygen consumption decreased 50% at 26°C, 80% at 20°C and 90% at 16°C. In our experiments, when the ascending aorta was interrupted under severe hypothermia, the cardiac muscles became bright red due to maintenance of the coronary circulation. After several heart beats a minute lasting for 20–30 minutes (the longest continued 70 minutes), it converted to ventricular fibrillation. In case the inflow tract was also interrupted, the cardiac muscles became immediately bluish, converted to ventricular fibrillation and stopped in cardiac standstill. All hearts were, however, successfully resuscitated.

In those cases with reanastomosis of the ascending aorta, permanent survival could not be obtained. The main causes are bleeding from the anastomosis in face of enough mobilization of the aorta. Especially, it is difficult to perform posterior anastomosis by twisting the aorta. In dogs the ascending aorta is very fragile and difficult to suture which is shown by the wide range of time for anastomosis (20–50 minutes). As anastomosis was securely made, no bleeding was observed during cardiac massage. Massive bleeding was, however, observed after defibrillation by electric shock. There was no way to prevent bleeding from the posterior wall.

Summary

In case the ascending aorta is interrupted, the clamped site will rupture prior to heart failure due to rapid overload on the heart regardless under normo or hypothermia.

Under severe hypothermia, it will, however, tolerate long cessation due to remarkable decrease of burden on the heart. Most of the cases

could be successfully resuscitated after the aorta alone is interrupted or the inflow and outflow tract are both obstructed at the same time. In case the ascending aorta is divided and reanastomosed, performance of operation is difficult and the main fatal cause is massive bleeding from the anastomosis.

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An Experimental Study on Transplantation of the Aortic Arch

Part II. Transplantation of the Aortic Arch under Severe Hypothermia (Rectal Temperature 18.5°-14°C)

By

Takao Takahashi
(高橋 孝雄)

From the Department of Surgery, Tohoku University, Faculty of Medicine, Sendai. Director: Prof. S-T. Katura

(Received for publication, April 22, 1957)

The author reported in the previous communication that under severe hypothermia, the heart could be successfully resuscitated after long cessation of the blood flow from the heart. In case the ascending aorta was divided and resutured, the technique was difficult. From these experimental results, considering less tension at the anastomosis and easy technique, transplantation of the aortic arch was carried out.

Experimental

Method

Anesthesia to obtain severe hypothermia
Same as that described in part I.
Cardiac resuscitation and warming
Same as that described in part I.
Operation
The chest was opened by a large incision encircling the scapula at the left 4th intercostal space. The descending thoracic aorta was mobilized and the superior, occasionally the 2nd intercostal artery was divided and ligated. The left brachiocephalic artery, then ductus Botalli was cut. The right brachiocephalic artery was freed, avoiding bilateral pneumothorax and finally the ascending aorta was dissected. Incising the pericardium longitudinally, the fat tissues at the reflecting portion of the pericardium were removed to completely free the aorta just above the aortic valve. A piece of tape was passed around the vagus and phrenic nerve to preserve them. Following interruption of the blood flow, clamps were placed on the right brachiocephalic artery and the descending thoracic aorta, and the aortic arch was removed en masse. With completion of anastomosis, the clamp on the ascending aorta was removed,

and the heart was compressed several times. This procedure was done for the purpose to prevent air emboli, test the amount and degree of blood loss at the anastomosis. Postoperatively, rewarming and cardiac massage were both done at the same time. Prior to closure of chest, the pericardium was roughly sutured. Chest drainage tube was inserted before chest wall was sutured. Postoperatively, energetic chemotherapy was instituted.

Cessation of blood flow (*Table I*)

8 cases were used with interruption of the outflow tract, the aorta. In case interrupting the aorta, with consideration of its fragility, a Satinsky's clamp loosely covered with rubber was used.

A strong silk was tied on its tip to prevent mobilization of the clamp. One showed ventricular fibrillation during mobilization of the aortic arch.

Inflow tract and the aorta were both interrupted in 10 cases. The heart was rotated out intrapericardially and Nelaton's catheters were used to constrict the inferior vena cavae. Clamps were not used. The obstruction was released from the inflow tract in the order given.

Material for transplantation

70% alcohol preserved homografts were used in 17 cases (Fig. 1.).

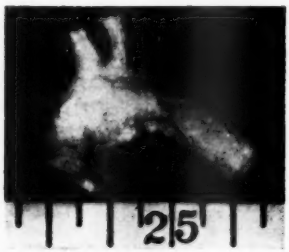

Fig. 1. Material used for transplantation. (70% alcohol preserved homograft)

Aorta with same diameter was selected. The left brachiocephalic artery was ligated at its origin beforehand. In one case, polyvinyl sponge was heated to make it poreless and made after an aortic arch exclusive of the left brachiocephalic artery with cuffs on both ends.

Anastomosis

Anastomosis was performed from the ascending aorta, the right brachiocephalic artery, and the descending thoracic aorta in the order given. First the anterior wall, then the posterior wall was sutured by a continous over and over stitches with 2 supporting sutures on both corners. Black silk No. 3 were used. 10% sodium citrate was injected into the inside to prevent clot formation. The anastomosis of the ascending aorta

TABLE I

Results of Transplantation of the Aortic Arch under Severe Hypothermia
(70% Alcohol preserved Homograft)

Dog No.	Body weight (kg.)	Cessation of blood flow		Cardiac resuscitation		Patency of graft		Survival period (hrs.)	Cause of death	Notes
		Outflow tract	Inflow tract	Success	failure	Yes	No			
80	12.5	+	−	+	−	+	−	2	Hemorrhagic shock	Polyvinyl sponge graft used.
84	13.2	+	+	+	−	+	−	2×24	Heart failure	Wrapping with "Amylan" cloth.
87	16.0	+	+	+	−	+	−	6×24	Empyema thoracis	
91	18.2	Cardiac arrest during cooling		+	−	+	−	1	Bleeding	Rupture at clamped site of the ascending aorta.
93	11.5	+	−	+	−	−	+	10	Death from shock	Thrombus of the descending aorta.
97	14.5	Cardiac arrest during cooling		+	−	+	−	1.5	Heart failure	Myocardial bleeding.
99	14.4	+	−	+	−	+	−	4×24	Heart failure	Bilateral pulmonary atelectasis.
101	12.0	Cardiac arrest during cooling		+	−	+	−	6	Pulmonary collapse	Bilateral open chest.
103	12.7	+	+	+	−	+	−	5×24	Empyema thoracis	
104	16.0	+	−	+	−	+	−	9×24	Rupture of anastomosis	Anastomosis of the descending aorta.
105	14.0	+	+	+	−	+	−	0	Filariasis	
106	16.0	+	+	−	−	−	−	14×24		Died during angiocardiography.
108	19.5	+	+	+	−	−	+	4	Paralysis of respiration	Thrombus of the right brachiocephalic artery.
109	12.0	Ventricular fibrillation at op.		+	−	+	−	2×24	Pulmonary collapse	Bilateral open chest.
110	25.0	+	+	+	−	+	−	2	Suffocation	Ice water in lung during cooling.
111	15.0	+	+	+	−	−	+	1.5	Paralysis of respiration	Thrombus of the right brachiocephalic artery.
112	18.0	+	+	−	+	+	−	0		
114	16·0	+	+	+	−	+	−	2	Paralysis of respiration	Myocardial bleeding.

was made with rough interval (1.5–2 mm.), adapting the intima closely.

Results

5 of 18 dogs (Nos. 87, 99, 103, 104 and 106) survived 4–14 days, while the others died within 2 days. The cause of death is described in each case.

In spite of no bleeding during cardiac massage after transplanting a graft made of polyvinyl sponge in No. 80, hemorrhage occurred from the entire graft after defibrillating the heart. In 84 "Amylan" was wrapped around the anastomosis of the ascending aorta to prevent bleeding. It died on the following night despite recovery of conciousness, movement of legs and reaction to pain stimuli. Dog Nos. 87 and 103 showed appetite on the 2nd day, walked on the 3rd day and reacted to petting. They died from empyema on the 5th and 6th postoperative day, respectively, despite energetic chemotherapy. Fig. 2 shows the re-

Fig. 2. Removed specimen in dog. No. 87.

moved specimen of No. 87. Operation was immediately started in No. 91, because of cardiac arrest during cooling at rectal temperature 17°C. Due to rough handling of the anastomosis of the ascending aorta, the clamped site was injuried, which caused fatal bleeding an hour later. Though No. 93 recovered from anesthesia, it died 10 hours later with spastic paraplegia of the hind legs. At autopsy, thrombus at the anastomosis obstructed the descending thoracic aorta. In Nos. 97 and 114, myocardial bleeding occurred during cardiac massage. Though it was successfully resuscitated, it died within a short time. No. 99 showed urinal and fecal excretion, started to walk on the 3rd day with no neurological disturbance, but died on the 4th day due to general weakness of the body. Nos. 101 and 109 both died 6 hours later and on the 2nd day, respectively. Both showed severe pulmonary collapse which was caused by bilateral pneumothorax during operation. Nos. 104 and 107 both showed a smooth postoperative course. However, the former died sud-

denly by rupture at the descending thoracic aorta on the 9th day, while the latter succumbed to death on the 14th day during angiocardiography with rapid injection of Urocolin 15 cc. into the left ventricle under intravenous anesthesia with Ravonal. Good patency of the graft was recognized (Fig. 3). The entire course of ECG is reproduced in Fig. 4, which shows return to normal tracings on the 12th postoperative day. No. 105 showed normal heart beat only to become weak again, which didn't respond to injection of 2% calcium chloride. At autopsy, the ventricle and atrium were both filled with filaria. Nos. 108 and 111 died from respiratory arrest prior to cardiac standstill. Autopsy revealed thrombus obstructing the aorta at the anastmosis of the brachiocephalic artery.

Fig. 3

Fig. 4

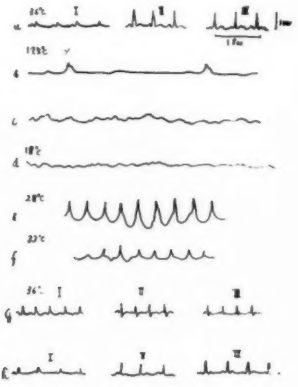

Fig. 3. Angiocardiography 14 days after transplantation of the aortic arch in dog No. 106. (sign ↑ in schema shows anastomosed part).
Fig. 4. Electrocardiographic tracing in dog No. 106.

Describing the patency at the anastomosis, 3 of 18 (Nos. 91, 111 and 108) showed obstruction of the lumen, of which 2 showed the smallest brachiocephalic arteries. In dog No. 108, the intima was injuried at the clamped site of the brachiocephalic artery (bulldog clamp was used in this case), from which extended a thrombus (Fig. 5). The remaining one showed thrombus at the anastomosis of the descending thoracic aorta. No obstruction was showed at the anastomosis of the ascending aorta. The remaining 15 were all patent.

Observing the relationship of cessation of cardiac circulation with the time for anastomosis, the shortest obstruction was 60 minutes, while the longest 140 minutes, average 88 minutes at rectal temperature 14°–18.5°C. The time for anastmosis was 50 minutes the shortest, while 85

Fig. 5. Injury of intima at the clamped site of the right brachiocephalic artery (↑) in dog No. 108.

minutes the longest, average 60 minutes. The time to anastomose the ascending aorta required 14 to 47 minutes. The marked difference was due to resuture the frequent tear of the fragile aorta (Table II).

TABLE II

Relationship of Time for Anatomosis and Cessation of Blood Flow of Heart

Dog No.	Rectal temp. (°C)	Time of interruption (min.)	Time for anastomosis (min.)
80	17.0	84	61
84	18.0	82	70
87	17.0	98	85
91	16.5	105	62
93	14.0	86	65
97	14.5	140	50
99	15.0	93	66
101	16.0	100	58
103	17.0	85	60
104	18.5	65	52
105	16.5	82	50
106	17.5	60	52
108	17.0	70	55
109	17.0	65	54
110	16.3	103	65
111	16.0	65	50
112	15.0	80	65
114	18.0	105	50

DISCUSSION

In 1955, Cooley *et al.*[11] reconstructed the blood vessel after resecting

an aneurysm of the ascending aorta with aid of a shunt. They pointed out the significance of blood to the brain and rapid increase of burden on heart following interruption of the ascending aorta. The following points were suggested to solve this problem.

1. In order to divide the aortic arch, shunts should be used to diminish increase of load on the heart, and prevent interruption of blood flow to the brain and peripheral organs, or the interruption should be limited as short as possible.

2. With use of hypothermia, both decrease of cardiac output and tissue oxygen consumption are obtained.

When shunt is used to prevent overload on the heart, tubes with various diameters are used. Izant *et al.*[2] demonstrated that in dogs shunt having inner diameter 5 mm. will permit 80% of blood flow to the periphery without inducing anoxia to the distal vital organs. Stranahan *et al.*[3] reported without definite conclusions that 10 mm. would be the minimum diameter required for shunts in clinical cases. In a few cases, the author used shunts under hypothermia as Satinsky reported.[4] We used the back flow from the left ventricle caused by artificial mitral insufficiency, but all failed except those with inner diameter 6–8 mm. When this method is used, the technique is complicating, shunts and clamps will obstruct the operative field, danger of massive bleeding is imminent, and fine technique is required, due to limit of cessation of blood flow. According to Satinsky,[4] the ascending aorta must be anastomosed within 11–20 minutes. As the ascending aorta is fragile and heart beat is strong, the danger to injure the blood vessel by overload on the heart can't be denied. According to Alley *et al.*,[5] completion of procedure was obtained in 9 out of 20, of which 5 of 11 were lost when shunt was sutured to the ascending aorta.

On the other hand, hypothermia has been applied in vascular surgery. Reports to apply hypothermia to prolong the cessation of the thoracic aorta have been published.[6] However, due to frequent occurrence of ventiricular fibrillation, severe hypothermia below 25°C seems to be discarded for use. Watanabe *et al.*[7] at this clinic have been studying severe hypothermia for 2 years, who have reported that ventricular fibrillation is not a serious problem under severe hypothermia, if appropriate method for resuscitation is at hand. They have been successful in an epoch making experiment to resuscitate a dog which had interruption of blood flow to the brain for 2 hours and 40 minutes, without heart beat and respiration. No serious postoperative sequelae was found on ECG, EEG, histology (heart, brain and other vital organs) and neurology. Though it is repeated again, applying severe hypothermia, the author devised a new method to transplant the aortic arch directly under long

cessation of cardiac circulation with arrested heart action, which is an entirely different method using various shunts by others. Hardin[8] lost 9 of 11 within a short time, of which 7 were in cardiac failure with arrhythmia, heart dilatation and ventricular fibrillation during operation. In case shunts are used, it seems to be difficult to restore normal rhythm when ventricular fibrillation occurrs, and to maintain effective blood pressure, which are both disadvantages of using shunts.

As described before, the time for anastomosis is 60 minutes, while that of obstruction of the heart flow 88 minutes. This difference shows that anastomosis can be done with leisure. In case of clinical application, because of unavoidable prolongation of time for anastomosis due to the anatomical difference in dog from human beings, it is the greatest advantage of using severe hypothermia.

Severe hypothermia is studied by Gollan *et al.*[9] and Schaefer *et al.*[10] in the U.S.A. and Watanabe *et al.*[7] in Japan. It is, however, not yet applied in clinical cases, but its clinical application is within near future. The possibility of applying transplantation of aortic arch which author experimented on clinical cases is demonstrated. This method will contribute to the advance of vascular surgery together with the conventional method using shunts.

Summary

The author transplanted the aortic arch with homologus grafts under long cessation of the blood flow from the heart as one of the applications of severe hypothermia which is presently studied by Watanabe *et al.* at this clinic. The blood flow of the heart was interrupted for a long time, 60–140 minutes, at rectal temperature 18.5°–14°C. Five of 18 survived the operation with successful outcome. Anastomosis could be carried out without tension and fear of massive blood loss, and it had the advantage to perform careful operation.

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Indirect Retinal Induction in Various Regions of the Human Retina

By

Eiichi Onodera

(小野寺 栄一)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

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Introduction

Visual contrast is a psychologically remarkable phenomenon, but it is difficult to account for this phenomenon in physiological terms, because there was no method available to measure the physiological process underlying it. Motokawa[1] showed that the color-effect manifests itself in the temporal pattern of electrical excitability of the retina following illumination. Utilizing this method, Motokawa[2] succeeded in exploring a retinal process to be correlated with the visual contrast and designated it retinal induction. There are two kinds of retinal induction, that is, direct and indirect retinal induction. The former represents a correlate of successive contrast, and the latter a correlate of simultaneous contrast. Both phenomena are in character complementary to each other. They remain unaltered for a periode of about 20 seconds after removal of the inducing light, and then decrease gradually to disappear in 30–40 seconds. This phenomenon was found not only in the human eyes, but also in animal retinas,[3),4)] and further it was shown that the retinal induction is caused not only by colored light, but also by intermittent white light,[5)] by alternating currents[6)] and by direct currents of various forms.[7)] The capacity to produce retinal induction is different from part to part in the retina. As to the regional difference of direct induction some experiments were made by Kurosawa and Katayama.[8)] In the present experiment a systematic survey on the spatial distribution of indirect induction was undertaken with special reference to the blind spot.

Experimental

Method

The method used was essentially the same as that employed by Motokawa, and the details of the procedures are described in previous papers.[1),9)]

The experiment was carried out in a dark room after a preliminary dark adpatation of about 20 minutes. A single constant current pulse of 100 msec. in duration was applied to the eye through a pair of silver electrodes 2×1.5 cm.^{2} in size, one (the cathode) placed on the forehead slightly above the eyebrow and the other (the anode) on the homolateral temple of the subject. The pulse was produced by Motokawa's microswitch stimulator. The index of excitation was the least perceptible electrical phosphene. First, the electrical threshold for the dark-adapted eye was measured in such a manner that, starting with a voltage sufficient to cause a distinct light sensation, the stimulating voltage was reduced step by step until the subject could no more distinguish an electrical phosphene from the background of intrinsic light of the retina. Near the threshold comparison procedures[10] were used which consisted in comparison of the effects of the test stimulus and the control stimulus of zero voltage. Then the excitability after an illumination was determined in a similar manner. The method of illumination was as follows. An opaque glass disc of 1.2 cm. in diameter (2° in visual angle) was illuminated from behind with spectral light. The spectrum used in this experiment was adjusted to equal energy. The white test-light was always 90 lux in intensity and its size was 10 minutes of arc in visual angle. The white light was made to fall on a part adjacent to the retinal area preilluminated by the colored light. The distance from the margin of the white test patch to the margin of the colored inducing patch was fixed usually at 1.5 mm. The distance between the patch and the subject's eye was always 34 cm. The effect of light was expressed in terms of ζ which was defined by the formula: $\zeta = 100(E-E_0)/E_0$ where E and E_0 denote electrical excitabilities (reciprocals of thresholds) measured with and without preillumination respectively. The measurement of indirect retinal induction was carried out at various points on the horizontal meridian on the nasal side of the fovea (on the temporal side in the visual field). As the positions of the illuminating patches were fixed, a fixation point, a faint red light, was placed at various distances from the patches.

Results

Indirect retinal induction caused by spectral lights of various wavelengths

The excitability curves obtained at various parts of the retina are shown in Figs. 1 and 2. In these Figs., ζ values are plotted as ordinates against time in seconds from the end of illumination to electrical stimulation as abscissae. The data concerned with red, green, yellow and blue lights are denoted by R, G, Y and B respectively. In the same Figs., the excitability curve for the white test light alone is represented by a dotted curve connecting crosses. The curves marked by empty circles and empty

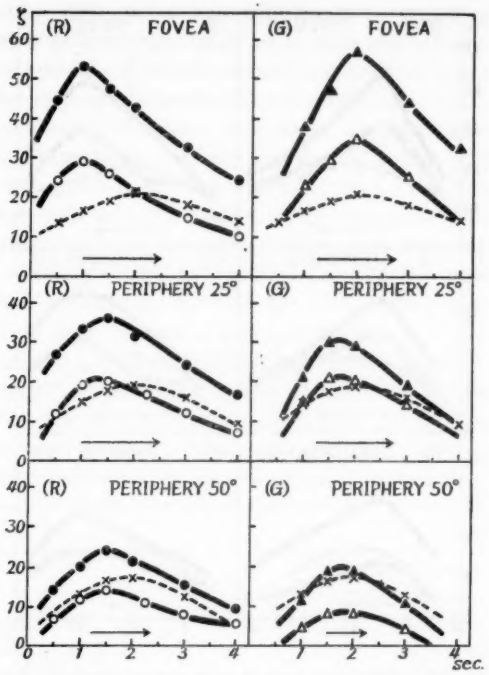

Fig. 1. ζ-time curves obtained at various parts of retina. Ordinates: percentage increases of electrical excitability above resting level. Abscissae: time in sec. from end of preillumination to electrical test stimulus. Curves marked by empty circles and empty triangles refer to red (R, 650 mμ) and green (G, 530 mμ) lights respectively. Curves connecting solid circles and solid triangles represent induction curves, that is ζ-time curves for red and green lights each succeeded by white test light. Dotted curves connecting crosses represent control curve for white test light alone.

triangles are the excitability curves obtained by preillumination with colored light alone for 2 sec. The curves marked by solid circles and solid triangles refer to retinal induction. To begin with, regional differences in the excitability curves for colored lights alone will be mentioned. The ζ-time curves for red, yellow and green lights have a maximum respectively at 1, 1.5 and 2 sec. after cessation of the preilluminating lights at the fovea. The curve for blue light shows two maxima at 1 and 3 seconds. The first elevation of the curve for blue light is much smaller than the second. The crest times of the curves obtained with the yellow and the blue lights do not change from the fovea to the periphery. But the crest time for the red light becomes longer, and that for the green light

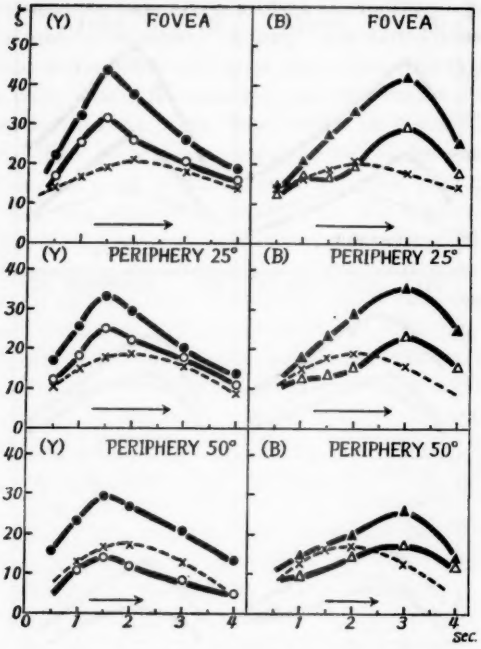

Fig. 2. ζ-time curves obtained at various parts of retina. Explanation as in Fig. 1. Y and B refer to yellow (580 mμ) and blue (470 mμ) lights.

becomes shorter, both converging into 1.5 sec. with increasing eccentricity from the center of the retina. In other words, the crest times for these colored lights become equivalent to that for yellow light. It is well known[11] that as moved from the fovea towards the periphery a red patch appears first orange, then yellow until it becomes colorless, and a green patch appears first yellowish-green, then yellow until it changes to white. The data shown above seem to be correlated with this psychological fact. In the previous paper by Motokawa,[1] it was shown that three kinds of color processes, red, green and blue, exist at the fovea and four kinds of color processes, red, yellow, green and blue, exist outside the fovea and that these color processes are characterized by their own crest times. The red and the green processes develop fully at the fovea, but become weaker than the yellow and the blue processes at the periphery. The same relation can be seen also in the data obtained by Motokawa[12] and by Oikawa.[13] Next, indirect retinal induction at various parts of the retina will be described on the basis of the data shown in Figs. 1 and 2. The

curves marked by solid circles and solid triangles were obtained by illuminating first with colored light and then with the white test light at an interval of 2 seconds. ζ values were measured at different times after exposure to the successive stimuli, colored and white. The exposure times of both stimuli were 2 seconds. The white test light illuminated a point of the retina just outside the area preilluminated by the colored light. The crest times of induction curves were 1, 1.5, 2 and 3 sec. respectively at the fovea, when red, yellow, green and blue lights were used as inducing light. The shape of these curves is the same as that of the curves for colored lights alone and is complementary in character to that of the curves of direct induction which would be obtained by illuminating one and the same retinal area with both colored and white light in succession. The curve of indirect induction is decidedly higher than that for the white light alone.[14] No regional difference could be found in the crest times of the induction curves for yellow and blue inducing lights, but the crest times were found to converge into 1.5 sec. with increasing eccentricity when red and green lights were used as inducing light. The curves shown in Fig. 3, refer to the spatial distribution of the intensity of indirect retinal induc-

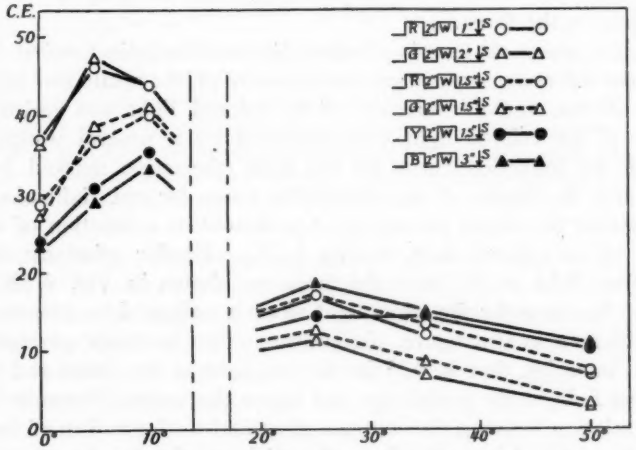

Fig. 3. Spatial distribution for indirect retinal induction. Abscissae; distances from fovea in visual angle (nasal side of the left retina). Distribution curves are interrupted corresponding to blind spot. Procedure for experimentation is shown in insets, where R, G, Y, B, W and S represent red, green, yellow, blue, white lights and electrical stimulus respectively.

tion, or the contrast effect which means the difference between the induction curve and the curve for the white test light alone. The full curves marked by empty circles, solid circles, empty triangles and solid triangles indicate

the contrast effects by red, yellow, green and blue lights respectively. In the neighborhood of the blind spot whose limits are indicated by two vertical lines, retinal induction suffers from qualitative and quantitative alteration, so that the detail will be retained for further report. The intensity of retinal induction is maximal at 5° from the fovea for red and green lights, and at 10° for yellow and blue lights. The curves for the red and the green lights were drawn with the values measured at 1 and 2 seconds respectively which correspond with the crest times of the respective curves at the fovea. In view of the fact that the curves for the red and the green lights have a crest at 1.5 seconds at the periphery, distribution curves were constructed with the values measured at 1.5 seconds. The results are shown by the broken curves in Fig. 3 which show a maximum at 10° from the fovea, in a manner similar to the distribution curves for yellow and blue lights. It is to be noted that the contrast effects of red and green lights are greater in the parafoveal region, but smaller in the periphery than those of yellow and blue lights. The result mentioned above is similar to the findings by Kurosawa and Katayama[8] about the direct retinal induction and may be correlated with the well-known psychological fact that the color contrast can be perceived more easily in the parafoveal region than in the fovea.

Relation between the intensity of colored light and the indirect retinal induction

In the following experiment the intensity of the white test light was fixed at 90 lux, and the intensity of the colored light was varied. The intensity of the colored light was controlled by a neutral wedge. The height of the induction curve for red light (the curve marked by solid circles) and the height of the excitability curve for red light alone (the curve marked by empty circles) are represented as a function of log. intensities of the colored light in Fig. 4 (A). Similar relations obtained with yellow light at 10° from the fovea are shown in Fig. 4 (B). The height of the curve for the white light alone is indicated by a broken line. As can be seen in this figure, the contrast effect becomes perceptible at a critical intensity, that is, 0.7^8 for the red light at the fovea and 0.7^6 for the yellow light at the periphery, and above the critical intensity there is a linear relation between the contrast effect and the logarithm of intensity. The higher critical intensity for yellow light might be due to its lower degree of saturation.

The indirect retinal induction in the neighborhood of the blind spot

Next, the indirect retinal induction in the neighborhood of the blind spot was studied. As an example the data obtained with yellow inducing light are shown in Fig. 5. The yellow patch used was 20′ in diameter and the white patch 10′ in diameter. The distance between the margins of both patches was 0.5 mm. The duration of the yellow and the white

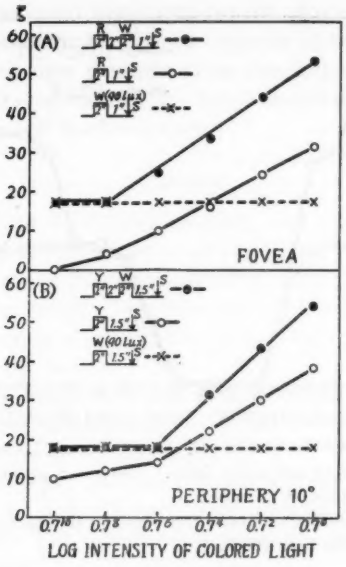

Fig. 4. Relations between ζ and logarithm of intensity of colored light. The highest intensity used, 0.7 was 350 times as high as the threshold intensity measured at the dark-adapted fovea with red light (650 mμ) of 2° in visual angle. Curves connecting solid circles were obtained with pair of stimuli, red-white in A and yellow-white in B. Curves marked by empty circles were obtained with red light in A, and with yellow light in B. ζ values for white light of fixed intensity 90 lux are shown by broken lines.

light was 2 seconds. The two photic stimuli were separated by an interval of 2 seconds. ζ values were measured at 1.5 sec. after termination of the white test light. As can be seen in Fig. 5, the distribution of ζ values is not symmetrical with respect to the blind spot; at the blind spot ζ values are zero and increase steeply towards the fovea, but not so steeply towards the periphery. A remarkable step can be seen at 21° from the fovea. Between 13° and 21° the distribution curve for induction coincides with that for the white test light (the broken curve), and this fact indicates that no retinal induction takes place within this retinal area. The blind spot concerning light sensations agrees with the area within which ζ values are zero, and this area is enclosed with a zone within which no color induction takes place. The zone without induction extends from 17° to 21° on the peripheral side of the blind spot, and from 14° to 13° on the foveal side. The subject reported that the yellow light looked colorless within the zone.

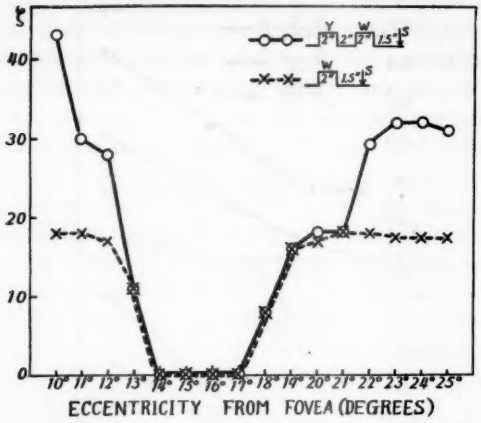

Fig. 5. Spatial distribution of ζ values measured 1.5 sec. after successive presentation of yellow and white lights. Abscissae: distances from fovea in visual angle. Ordinates: ζ values.

Dotted line connecting crosses represents ζ value measured 1.5 sec. after illumination of white light (90 lux).

Discussion

The present investigation together with the experiment by Kurosawa and Katayama indicates that the capacity to produce retinal induction is maximal at about 10° from the fovea. Aizawa and Katayama[15] investigated lateral summation and inhibition in the human retina, and found that the strongest inhibition takes place at about 10° from the fovea. In their experiment the brightness of a spot of light was depressed by presentation of a second spot in the neighbourhood of the former. This depressant effect was found maximal at about 10° from the fovea. The inhibition was shown also in another form; Aizawa and Katayama found that in this retinal area the ζ value for two spots of light was decidedly lower than that for a single one. These authors ascribed such remarkable spatial inhibition to the well-developed lateral nervous connections in this retinal area. The indirect retinal induction represents a sort of spatial interaction. Therefore it must be based on some anatomical lateral connection in the retina. It is important that both phenomena, inhibition and induction are more remarkable at the periphery than at the fovea where lateral connections are said to be scarce. Motokawa[14] suggested that retinal induction may be related to a postinhibitory rebound. The close relation between retinal inhibition and induction may be understood from this point of view. Finally it must be mentioned that there is a color-blind zone around the blind spot. Within the zone responses to white light

can be obtained in terms of a light sensation or of positive ζ values, but no retinal induction can be produced by colored light in this zone. The extent of the colorless zone depends upon the intensity and visual angle of colored light and its wavelength in such a complicated manner that the detail will be reported in further papers.

Summary

By the method of electro-stimulation, the magnitude of indirect retinal induction was measured at various parts of the human retina.

1. The magnitude of the indirect retinal induction was found greatest at about 10° from the fovea. This fact might be correlated with the psychological fact that color contrast can most easily be perceived in the parafoveal region of the retina.

2. The capacity to produce the indirect induction is great for red and green lights in the neighbourhood of the fovea, and for yellow and blue lights at the periphery of the retina.

3. The contrast effect increases in proportion to the logarithm of the intensity of colored light above a critical intensity, below which no induction takes place. The threshold of contrast effect is higher than the threshold of color effect.

4. Around the blind spot there is a zone in which no retinal induction takes place, but white sensations occur.

The author is greatly indebted to Prof. K. Motokawa, under whose guidance his investigation was performed, for his generous advice, and valuable suggestions throughout the course of the experiment and the preparation of the manuscript.

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A Sensitive Method for Detecting the Effect of Radiation upon the Human Body

By

K. Motokawa, T. Kohata, M. Komatsu, S. Chichibu,
(本川 弘一) (木幡 正) (小松 三夫) (秩父 志行)
Y. Koga* and T. Kasai*
(古賀 良彦) (笠井 武夫)

From the Department of Physiology and the Department of Radiology, Tohoku University, Sendai*

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Introduction

It is one of the most urgent problems to have a sufficiently sensitive and reliable method available for detecting the effect of radiation upon the human body. The physical methods available at present are extremely sensitive and reliable as well, but can tell nothing about biological effects. There are a number of biological methods to detect the effect of ionizing radiation, but it is generally difficult to apply them to human being. The generally accepted hematological methods are not sufficiently sensitive; a single or accumulated dose of tens or perhaps hundreds of roentgens may be needed for a positive response.[1)]

Motokawa, Umetsu and Kobayashi[2)] showed that the effect of a small dose of X-rays down to several hundred mr could be detected by their method of electric flicker. Umetsu[3)] provided evidence that the effect is a biological response of the retina. In this method the electrical threshold of the eye is measured, taking electrical phosphenes, a sensation of light to electrical stimulation of the eye, as an index. The threshold increased following temporal exposure to radiation and subsided in about 30 minutes approximately to the initial level. The threshold dose for this effect was found to be about 1 roentgen. The response is too temporary in marked contrast with the enduring effects revealed by other biological methods. In the present investigation, however, it has been elucidated that in the preceding investigation by Motokawa *et al.* a long-lasting effect was overlooked because of insufficient accuracy of the previous method.

The present investigation dates back to the following event in the Department of Physiology: One of the authors, T. K. was engaged in the study of visual physiology. In this research the effect of light upon the electrical

threshold was measured very accurately using the routine method in the department. T. K. was one of the best subjects for such measurements, for his electrical threshold was accurate and showed only little daily variations. Recently, however, he fell, from time to time, into an enormously confused state in which his threshold was found very high and unstable. Such a state, once started, used to continue for a few to several days. He was almost giving up his research, because the occurrence of the state was becoming more frequent. We sought for factors likely to be responsible for the state in question, but had no clue. We never thought of an effect of radiation until we were told that he sometimes carried out the fluorography of the chest at a certain hospital as a part-time physician there. Then a survey was made on the coincidence between the state and the fluorography, and it was confirmed that there was a certain correlation between them, and further that the effect was greatly reduced, but not entirely avoided by protecting the eyes with a lead plate. Suspecting the effect of scattered X-rays to be responsible for the state, we consulted one of the authors, Y. K. He doubted, however, the relationship in general, because it seemed to him that the scattered X-rays received by an observer in the usual fluorography would be too small in quantity to cause such a remarkable effect. He rather thought of some failure in radiological technique at the hospital and stressed the importance of careful control of doses. Thus cooperation was started between both departments of physiology and radiology. The dose of ionizing radiation was now controlled with the highest possible accuracy, and thus positive responses were obtained to such a small dose as a few mr.

The present method is based on the same principle as the previous work. The only difference consists in a great improvement in the accuracy of threshold determination; the rise in sensitivity amounting to 100–1000 times and confirmation of a long-lasting after-effect of momentary exposure depend solely upon the improvement in the technique for measuring electrical thresholds and careful control of experimental conditions. In the present paper, therefore, the method, especially the procedure for determination of thresholds, will be described in full detail.

Experimental

Method

1) *Apparatuses necessary for measuring electrical thresholds of the human eye*

A single rectangular pulse of 100 msec. in duration is applied to the human eye through a pair of silver electrodes of 2×1.5 cm.^{2} in size, placed one on the forehead and the other on the temple. The contact of the electrodes with the skin was secured by the use of electrode paste. The circuit for electrical stimulation is shown in Fig. 1. The contacts S_1 and S_2 are opened in succession at a

fixed interval of 100 msec. by means of a rheotome illustrated in the same figure. The duration of 100 msec. is about 2 times the utilization time of the rheobasic current. The strength of the stimulating current is controlled by a variable resistance R in the figure. Another constant resistor of 10 kilo-ohms serves to reduce

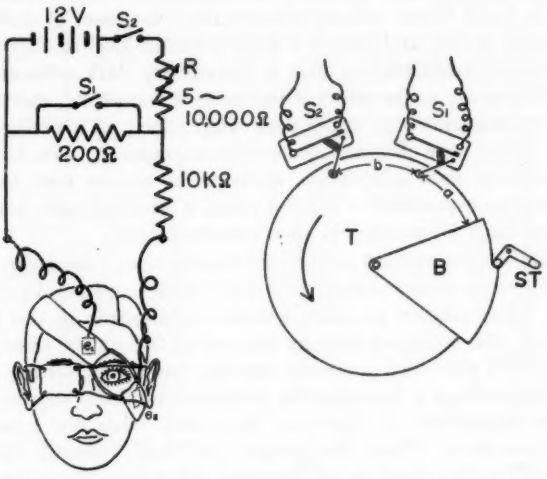

Fig. 1. Arrangements for measuring electrical thresholds of human eye. A rheotome is shown on right. B: Sector which serves to open switches S_1 and S_2 when it turns with T. S_1 and S_2: Microswitches. ST: Stop for B. T: Turning board of phonomotor. Further explanation in text.

the effect of variation of the skin resistance. We usually place the cathode on the forehead. A small resistance of 200 ohms is connected in parallel to the tissue, and the voltage applied to both ends of the resistance is calculated after the following formula:

$$v=\frac{12\times 200}{R+200} \text{ volts} \qquad (1)$$

As the contacts of the rheotome " microswitches " are used, which are opened by a revolving sector B and closed by their elasticity when B has passed under each switch. The sector B is put co-axially on a turning board T of a phonomotor and revolves with T by friction between B and T. The sector can, however, be brought into standstill by means of a stop ST, while T is turning. When the sector is released manually, it turns with the same rate as T. The switch S_1 is opened by the anterior edge of B, and after a certain interval the other switch S_2 is opened, the interval being determined by the distance between S_1 and S_2 and the revolving rate of B. When the trailing edge of B just passes S_1, this is closed by its elasticity. Thus the subject's eye is shunted so that it is never stimulated until S_1 is opened again. S_2 is closed likewise when the trailing edge

of B passes S_2, and thus a battery of 12 volts is switched in for the next stimulation. The sector B stops automatically after one revolution. In this way one has only to release the sector B to give a stimulus.

2) *The adaptation state of the eye*

The electrical threshold of the eye as determined with electrical phosphenes as an index is found lowest several minutes after the onset of dark adaptation, then rises rather rapidly and attains a fairly constant level in about 20 minutes. We usually make measurements after a preliminary dark adaptation of about 20 minutes. But so far as the effect of radiation is concerned, there is no reason to carry out experiments only under dark adaptation; in a preliminary experiment similar effects were obtained under light adaptation. We, however, prefer dark adaptation to light adaptation, because the former may be reproduced easily. During an experiment a fixation point, a faint red light, is used, because eye movement must be avoided in such measurements.

3) *Gradation of stimulating currents and determination of thresholds*

We usually start with a voltage of 2 volts which correspond to 1000 ohms of R (Fig. 1). Most subjects perceive a distinct phosphene to this stimulus. If the subject fails, the resistance may be reduced to 500 or 300 ohms, but it must be borne in mind that such a strong stimulus has a long-lasting after-effect.[4] The stimulating voltage is lowered (the resistance is raised) step by step in such a manner as exemplified in Table I. Successive trials are separated by an interval of 15 seconds. When the voltage is so much reduced that the subject finds it difficult to discriminate an electrical phosphene from the background of intrinsic light, he demands a comparison procedure. The procedure consists of delivering at an interval of several seconds in random order two stimuli, one of which is the stimulus in question, and the other a control one of zero-voltage. The subject is requested to answer the question which the strong stimulus is, and informed every time whether his answer is right or wrong. The same procedure is repeated with voltages reduced step by step until the subject can no longer discriminate both stimuli. Near the threshold the voltage is graded by about 1 per cent, because normal subjects can discriminate a difference of about 1 per cent. If the answer is wrong, the test with the same voltage is repeated, and if the subject fails twice out of three trials, the latest value is adopted as a threshold value. In these tests the subject is allowed to request a further trial when he thinks that his failure was due to some accidental disturbance of attention or due to wrong judgement in spite of a fairly distinct difference perceived between the effects of the two stimuli. In reality, however, such positive cooperation cannot always be hoped from all subjects. In the present experiment in which all the subjects were beginners, a further trial with a stimulating voltage reduced 5 per cent was made to be sure that no positive response appeared below the threshold obtained. Though rarely, successive positive responses were obtained, which indicates that the threshold once determined was not a true one (see Table I, the 3rd column).

The method mentioned above has been established after our long experiences of about 10 years and gives well-reproducible and reliable results, as will be

TABLE I

Examples of Protocol for Gradation of Stimulating Voltages

Values of resistance R in Fig. 1 are given in ohms. Correct answer is marked by plus sign, wrong answer by minus sign, and no or equal phosphene to both stimuli by circle.

N.H. (normal)		B.M. (X-ray gr.)		K.U. (X-ray gr.)		N.C. (X-ray gr.)	
R	Response	R	Resp.	R	Resp.	R	Resp.
1000	Distinct	800	Clear	1000	Clear	800	Weak
1500	〃	1000	Weak	1500	〃	1000	〃
1800	〃	1050	V. weak	1800	Weak	1100	〃
2000	Weak	1100	Faint	2000	〃	1200	Faint
2150	〃	1140	〃	2100	Faint	1240	T. faint
2250	Very weak	1180	Weak	2150	〃	Comparison	
2280	Faint	1220	Faint	2170	V. faint	1260	+
						1280	−++
2310	〃	1260	〃	2190	T. faint	1300	+
						1320	+
2340	Too faint	1300	V. faint	Comparison		**1340**	○++
						1360	−○
Comparison		1340	T. faint	2210	+		
				2230	−−	Step up 5%	
2370	+	Comparison					
2400	+	1360	+	Step up 5%		1440	○○
2430	+	1380	+				
2460	−++	1400	−++	2350	+		
2490	++	1420	+.	2380	+		
2520	○○	1440	+	**2410**	−++		
Step up 5%		1460	+	2440	−−		
		1480	+	2470	○		
2650	−○	**1500**	+				
		1520	−○				
		Step up 5%					
		1600	○−				

shown later. The slightest modification can reduce the reliability and accuracy greatly, as pointed out by Kohata *et al.*[4] Therefore it is desirable to follow this procedure as faithfully as possible, before any modification is made.

4) *Various factors affecting threshold measurements*

a) Resistance of the skin. The absolute value of threshold depends upon the electrical resistance of the tissue, especially the skin. The constant resistance of 10 kilo-ohms in Fig. 1 reduces this effect to some extent. Sweating lowers the resistance of the skin and consequently the threshold. It is, therefore, necessary to maintain the room temperature and ventilation constant.

The absolute value of threshold shows individual and daily variations even under well-controlled experimental conditions. It is, therefore, preferable to use some relative measure comparatively independent of the absolute value. As will be mentioned later, the standard deviation in per cent of the mean

threshold serves as an excellent indicator for the effect of radiation.

b) Attention and attitude of the subject. Weak phosphenes can be perceived only when the subject concentrates his attention to the phenomenon. It is sometimes difficult for a beginner to concentrate attention to a phosphene appearing in the periphery of the visual field. Electrical phosphenes are sometimes very similar to the intrinsic light of the retina, but have a characteristic that they appear in definite relation to the electric stimulus. In order to make discrimination of phosphenes easier, the subject must be informed of the moment at which the electrical stimulus is given. We give, therefore, at least two anticipatory signals; the one is a verbal signal which is given a few seconds prior to the electric stimulus, and the other the clicks from the switches. It is true that conditioned phosphenes may be evoked by these anticipatory signals alone, but they are avoided by the comparison procedure stated above.

c) Fatigue. Electrical phosphenes are diminished in intensity by fatigue. The method of electric flicker is based on this principle.[2] The effect of ionizing radiation as revealed by the present method is not specific to radiation, but very similar to the effect of fatigue. Therefore it is necessary to carry out experiments only in the normal, not fatigued state of the subjects.

d) Practice-effect. In some subjects the value of threshold and its fluctuation decrease as they become more practiced, but in others no remarkable practice-effect can be observed (see Tables II and III). If one session is devoted to practice, the practice-effect may practically be avoided.

5) *Dosimetry of ionizing radiation*

X-rays were measured by means of a Küstner's dosimeter. The intensity was controlled by varying the distance between the focus and the eye. The conditions of exposure were as follows: Voltage 70 kVp, current 3 mA, filter 0.5 mm. Al, exposure time 1 minute and distance 155 cm. for dose of 50 mr. Radium was used when the effect of radiation at a dose less than 50 mr was investigated. For doses of 1 mr and 5 mr, Ra of 1 mg was held at a distance of 20 cm. in front of the subject's eyes, exposure time being 3 and 15 minutes respectively. For greater doses Ra of 6 mg was used, the distance being fixed at 20 cm. The measurement by the Küstner's dosimeter was checked further by a pocket-chamber and with a milliroentgen-meter (Toshiba).

Results

A) *The effect of momentary X-ray exposure on the electrical threshold of the human eye*

The dose of X-rays used in this series of experiments was 50 mr. The subjects were 10 normal university students who were all beginners in such measurements. After a preliminary dark adaptation of about 20 minutes, electrical thresholds were measured 10 times after the procedure described above. The mean of the 10 values and their standard deviation were obtained. Then each subject was exposed to X-rays, and 10–20 minutes after exposure, similar measurements were carried out. The results obtained were consistent in all the cases. Similar measurements were performed on successive several days till

the after-effect of exposure apparently disappeared. The threshold increased after exposure, and far greater increases were found in the standard deviation. These changes subsided day after day. In some cases a second exposure was carried out several days after the first, and the same changes could be observed.

Strictly speaking, the decay of the threshold value itself or the standard deviation was not so steady in some cases, but it was found that the decay became very smooth in all cases, when the effect was expressed in terms of relative values of standard deviation. This measure has proved to be very useful not only for specifying the effect of momentary exposure, but also certain chronic effects found in persons occupationally exposed to radiation. Therefore this quantity, i.e. standard deviation in percentage of the mean, will be called the " phosphene index " and denoted by a symbol Pi. The phosphene index is defined by the following formula:

$$\mathrm{Pi}=\frac{100}{\mathrm{M}}\sqrt{\frac{\Sigma(\mathrm{v}-\mathrm{M})^2}{\mathrm{N}}} \qquad (2),$$

where v, M, and N denote each threshold voltage, the mean and the number of measurements, 10 in the present experiment, respectively.

The time-courses of Pi after exposure to 50 mr are summarized in Fig. 2. As can be seen in this figure, Pi decreases steadily with time. The time necessary for complete recovery could not be determined from this series of experiments, because the practice-effect mentioned above was not ruled out.

B) *The effect of radiation as a function of applied doses*

The maximum value of Pi following momentary exposure to ionizing radiation from Ra was measured on 18 normal subjects including 8 well-trained subjects from the Department of Physiology. The effect is shown as a function of the applied doses in Fig. 3, where Pi is expressed in terms of Pi_0, the pre-exposure value of Pi. As can be seen in this figure, the effect increases almost linearly with the logarithm of the dose applied for smaller doses than 50 mr. It is to be noted that such a regular relation has been obtained, although the points in the figure refer to different individuals. We may say definitely that ionizing radiation of a dose as small as 1 mr is effective. If the extrapolation of the curve is allowed, the minimum detectable dose, which is represented by the intersept on the dose-axis, is about 0.4 mr. This dose is said to be of the same order as the daily background radiation on the surface of the earth.

C) *Enduring effects as found in X-ray-technicians and physicians*

The phenomenon mentioned above is of great biological importance because of its extremely high sensitivity to ionizing radiation. But if it were a mere temporary effects having no connection with the cumulative effect, which is said to be characteristic of radiation, it would be a mere biological response comparable with the physiological response of the retina to visible light. In reality, the after-effect of momentary exposure endures for a few to several days or longer. There are few parallels to this phenomenon with regard to the duration of the after-effect.

The following series of experiments were undertaken to find any cumulative

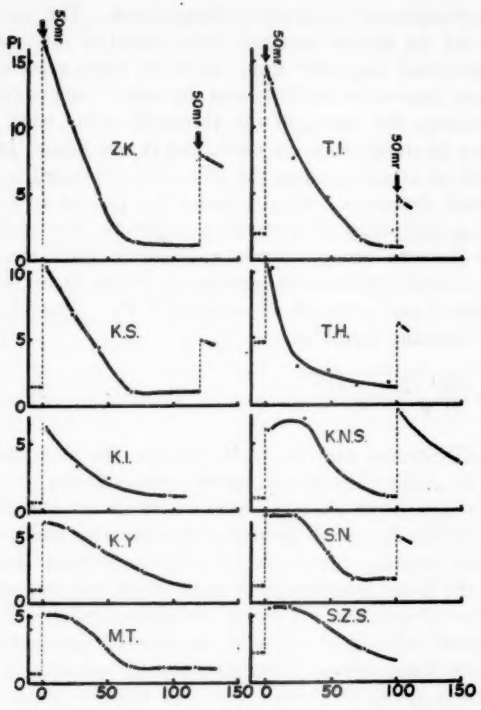

Fig. 2. Effect of X-ray exposure as a function of time in hours after exposure. Ordinate : Phosphene index or standard deviation in per cent of mean of 10 thresholds. Arrows indicate exposure. Subjects : Normal university students.

effects in persons occupationally exposed to ionizing radiation. The subjects were X-ray-technicians and physicians in the Department of Radiology and other hospitals, the number being 21. As control as many normal beginners, mostly university students, were used. In order to avoid any after-effect of the latest exposure, each subject was requested to have a vacation of a week at the shortest. Each subject had four sessions, 10 measurements in each. The protocols are summarized in Tables II and III. The phosphene index remained almost steady in most cases, but that in the first session was remarkably higher in some cases (practice-effect). Therefore the data of the first session were discarded in the following statistical treatment. A histogram was constructed from 63 values of Pi, and is illustrated in Fig. 4 A. The mean value of 63 Pi was $2.50 \pm 1.661\%$. Similarly another histogram B was constructed from the data of the normal group, the mean value of Pi being $0.98 \pm 0.346\%$. As can be seen in the figure, the phosphene index of the X-ray group shows a distribution quite different

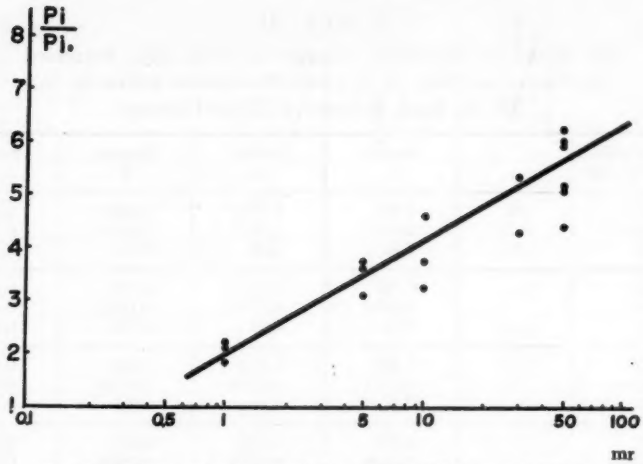

Fig. 3. Effects of ionizing radiation from Radium as a function of applied doses. Ordinate: Phosphene index with pre-exposure value as unit. Abscissa: Logarithm of dose in mr. All points were obtained from different normal subjects.

from that of the control group. There was no subject showing Pi greater than 2.5 in the control group, while the mean value of Pi was 2.5 in the X-ray group.

The histograms of the two groups overlap each other over a certain limited region of Pi. This fact may be correlated with the circumstance that some individuals in the X-ray group were quite normal not only concerning blood cell counts, but also as to other physical conditions. It is, however, to be noted that values of Pi smaller than 0.9 were found 21 in number in the normal group, while there was no such small value in the X-ray group. This is a fact suggesting that the capacity to discriminate electrical phosphenes is more or less impaired even in apparently normal subjects of the X-ray group. In this way a cumulative effect of radiation has been demonstrated by the present method.

For comparison, histograms of threshold voltages were constructed likewise and are illustrated in Fig. 5. The mean of the thresholds for the X-ray group was 1.686 ± 0.8907 volts, and that for the normal group 1.414 ± 0.4471 volts. As can be seen in the figure, the overlap of both histograms is far more remarkable for threshold voltages than for phosphene indices; the difference between both groups is hardly significant when threshold voltages are taken as an index. This is one of the reasons why we prefer the phosphene index to the threshold voltage itself.

D) *Summation of the effects of X-ray exposures and the duration of the after-effect*

In order to study the summation of the effects of exposures, two well-trained subjects were exposed to X-ray irradiation with a dose of 50 mr every day or every two days, and changes of Pi were observed for about 2 weeks. The results are illustrated in Fig. 6. As can be seen in this figure, the phosphene index

TABLE II

The Mean of Threshold Voltages in Volts (M), Standard Deviation in Volts (S. D.) and Phosphene Index in % (Pi) in Each Subject of Control Group

Name of subj.; Profession; Age		Session 1	Session 2	Session 3	Session 4
T.O. Student 25	M S.D. Pi	1.094 0.0417 3.81	0.994 0.0075 0.76	1.068 0.0075 0.70	1.026 0.0082 0.80
S.C. Student 25		0.748 0.0055 0.74	0.721 0.0044 0.61	0.701 0.0025 0.36	0.701 0.0038 0.54
N.T. Student 25		1.192 0.0271 2.27	1.173 0.0188 1.60	1.005 0.0060 0.60	0.840 0.0076 0.90
E.Y. Laborant 32		0.981 0.0116 1.18	0.820 0.0070 0.85	0.812 0.0084 1.04	0.787 0.0073 0.93
Y.O. Student 22		1.262 0.0424 3.36	1.173 0.0135 1.15	1.146 0.0126 1.10	1.554 0.0125 0.80
N.H. Student 22		1.154 0.0233 2.03	1.043 0.0080 0.77	0.901 0.0071 0.78	0.997 0.0079 0.79
M.A. Student 21		1.340 0.0202 1.51	1.337 0.0111 0.83	1.277 0.0106 0.83	1.163 0.0159 1.37
R.K. Student 23		0.980 0.0136 1.39	1.020 0.0097 0.95	0.847 0.0060 0.71	0.857 0.0042 0.49
O.S. Student 22		1.782 0.0199 1.12	1.543 0.0287 1.86	1.814 0.0261 1.44	1.691 0.0137 0.81
N.M. Student 22		2.201 0.0710 3.22	1.597 0.0198 1.24	1.646 0.0134 0.81	1.314 0.0102 0.78
G.S. Student 21		2.355 0.0156 0.66	1.409 0.0124 0.88	1.282 0.0083 0.65	1.259 0.0100 0.79
C.K. Student 20		2.185 0.0343 1.57	1.916 0·0230 1.20	1.780 0.0197 1.11	1.338 0.0096 0.72
S.I. Student 19		1.189 0.0128 1.08	0.857 0.0074 0.85	0.952 0.0100 1.05	0.961 0.0042 0.44

T.S. Student 22		1.756 0.0419 2.38	1.843 0.0293 1.59	1.823 0.0416 2.28	1.815 0.0267 1.47
G.M. Student 19		0.959 0.0113 1.18	0.954 0.0097 1.02	0.824 0.0049 0.60	0.765 0.0049 0.64
J.M. Student 23		1.931 0.0201 1.08	1.720 0.0245 1.43	1.678 0.0148 0.88	1.490 0.0180 1.21
S.T. Student 22		1.463 0.0154 1.05	1.501 0.0146 0.97	1.617 0.0226 1.40	1.825 0.0176 0.96
R.K. Student 22		1.722 0.0241 1.40	0.888 0.0075 0.85	0.813 0.0054 0.66	0.850 0.0068 0.80
T.S. Student 22		2.130 0.1400 6.56	2.024 0.0304 1.50	1.171 0.0224 1.91	— — —
K.M. Professor 53		— — —	0.596 0.0050 0.84	0.594 0.0046 0.79	0.593 0.0040 0.67

rises immediately after each exposure and subsides steeply, but never recovers to the preceding level. The effect of the next irradiation is added to the residual effect of the preceding one, and thus the curve of Pi rises slowly as a whole. So a striking resemblance is seen between this effect and the incomplete tetanus of the skeletal muscle. The total dose used was within the maximum permissible dose in both cases. Nevertheless it took a very long time for complete recovery. Two phases, acute and chronic, may be distinguishable in the curve of Pi. The acute phase lasts for a few days, and at the end of this phase the rate of change in Pi is slowed down remarkably. The recovery in the chronic phase was so protracted that it was not yet complete after 10 days from the latest exposure. It is likely that the cumulative effect as found in the X-ray group is based on such a protracted after-effect.

In any case the chronic effect is far less conspicuous than the acute one. The former is comparable with the tonus, and the latter with a contraction of the muscle. Because of the small magnitude the chronic effect tends to be overlooked when the accuracy of measurement is not sufficient.

The question was raised as to whether the acute response of persons constantly exposed to weak radiation was different in any respect from the normal one observed above. One subject H. E. of the X-ray group, whose resting value of Pi was 1.05, showed a value 1.91 when at work. Immediately after exposure to 50 mr the value increased to 5.02. Another subject K. U. with a resting value of 1.71 and a value at work 3.18 showed such a high value as 12.3 following exposure to 50 mr. These findings give, however, no conclusive answer to the question raised above. The question must be studied in further experiment.

TABLE III

The Mean of Threshold Voltages in Volts (M), Standard Deviation in Volts (S. D.) and Phosphene Index in % (Pi) in Each Subject of X-Ray Group

In remarks are given years of professional work, leucocyte count and days of vacation in order.

Name of subj.; Profession; Age		Session 1	Session 2	Session 3	Session 4	Remarks
Y.T. Physician 34	M S.D. Pi	1.640 0.0378 2.30	1.271 0.0150 1.18	1.143 0.0134 1.17	1.394 0.0158 1.13	8 years 5900 9 days
K.S. Technician 43		1.052 0.0268 2.58	1.223 0.0290 2.37	1.084 0.0173 1.59	1.148 0.0238 2.07	18 years 7400 9 days
N.W. Physician 28		2.640 0.1940 7.35	2·347 0.0500 2.13	1.806 0·0340 1.88	1.812 0.0696 3.84	3 years 5600 9 days
H.K.M. Physician 32		1.449 0.0324 2.24	1.227 0.0161 1.31	1.223 0.0273 2.23	1.196 0.0128 1.07	7 years 6500 9 days
T.K. Physician 29		1.058 0.0285 2.70	1.010 0.0099 0.98	0.948 0.0109 1.13	0.816 0.0091 1.12	3 years 4100 9 days
Y.K. Professor 56		2.151 0.0358 1.67	2.688 0.0469 1.74	2.704 0.0511 1.89	1.695 0.0260 1.54	20 years 3800 9 days
G.N. Physician 32		1.273 0.0112 0.88	1.219 0.0312 2.56	1.529 0.0328 2.15	1.434 0.0180 1.26	3 years 3100 6 days
S.S. Technician 40		5.301 0.2490 4.69	5.292 0.3740 7.07	3.420 0.2810 8.21	5.570 0.5490 9.85	22 years 5400 11 days
H.E. Technician 31		1.138 0.0107 0.94	1.072 0.0115 1.07	0.935 0.0080 0.85	0.789 0.0098 1.24	8 years 8300 7 days
K.U. Physician 29		2.277 0.0660 2.90	1.149 0.0194 1.69	0.923 0.0178 1.93	0.895 0.0136 1.52	2.5 years 6700 6 days
B.M. Technician 38		1.660 0.0716 4.31	1.451 0.0396 2.73	1.649 0.0306 1.86	1.398 0.0426 3.05	7 years 3400 7 days
H.H.M. Technician 45		1.199 0.0267 2.23	1.199 0.0386 3.22	1.256 0.0448 3.57	1.221 0.0313 2.56	21 years 3200 7 days

C.I. Technician 29		1.548 0.1500 9.69	1.802 0.0811 4.51	1.580 0.0446 2.83	1.950 0.1020 5.23	6 years 6300 7 days
N.C. Technician 45		1.008 0.0184 1.83	1.168 0.0271 2.32	1.083 0.0246 2.27	1.506 0.0459 3.04	28 years 5200 6 days
S.M. Technician 21		2.753 0.0689 2.51	3.255 0.0476 1.47	1.644 0.0229 1.39	2.543 0.0533 2.09	1 year 8700 6 days
S.K. Techniciah 31		4.084 0.0673 1.65	1.353 0.0302 2.23	2.120 0.1102 5.20	1.146 0.0221 1.93	8 years 7000 7 days
M.K. Technician 36		1.870 0.0734 3.92	1.839 0.0471 2.56	1.633 0.0334 2.04	1.361 0.0220 1.62	8 years 6200 6 days
H.S. Technician 23		2.644 0.0408 1.54	2.058 0.0286 1.39	2.231 0.0531 2.38	2.331 0.0742 3.19	4 years 6000 6 days
H.T. Physician 41		3.015 0.0826 2.74	2.965 0.0625 2.11	1.478 0.0706 4.78	1.151 0.0174 1.51	8 years 6000 7 days
M.I. Physician 43		3.184 0.0962 3.02	1.956 0.0585 2.99	1.662 0.0558 3.36	1.153 0.0350 3.02	10.5 years 4000 6 days
S.T. Technician 36		1.388 0.0288 2.08	1.337 0.0223 1.67	1.368 0.0279 2.04	1.333 0.0215 1.61	13 years 3800 4 days

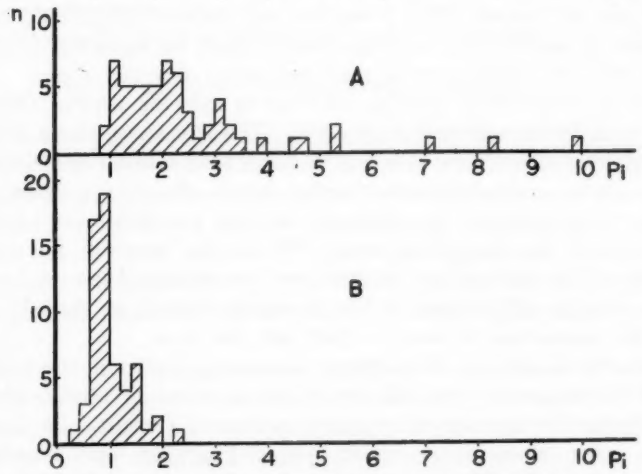

Fig. 4. Histograms of phosphene indices. A and B refer to X-ray group and control one respectively. The former consisted of 21 X-ray-technicians and physicians who were not at work before and during experiment.

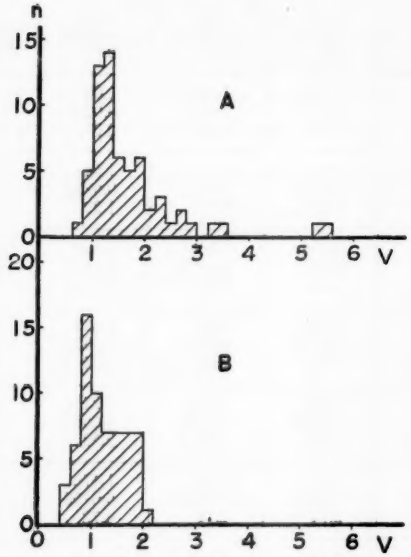

Fig. 5. Histograms of threshold voltages. A and B refer to X-ray group and normal control respectively.

Discussion

The acute effect is clear-cut and sufficiently sensitive to ionizing radiation, while the chronic effect is obscure and perhaps insensitive; it seems likely that thousand times as large doses would be necessary to cause a chronic effect as conspicuous as the acute effect observed above.

It is an interesting question whether or not the present effects are related with the hematological changes. The leucocyte counts of the X-ray group were either normal or a little lower than normal (see Table III). The phosphene indices measured in the chronic phase were either normal or higher than normal. In this sense we may say that some correlation exists between the two phenomena. We made, however, no statistical treatment of the correlation, because our hematological material was not uniform enough with regard to the measuring technique, the laboratories where the measurements were carried out and date.

As to the sensitivity of the blood to ionizing radiation, the results obtained hitherto are too contradictory to one another. Wakabayashi *et al.*[5] observed that the number of lymphocytes showed a statistically significant decrease after momentary exposure in radiography and fluorography (received doses 16–60 mr), and that the effect was most marked after 30 minutes and disappeared in 60 minutes. Helde & Wahlberg[6] re-

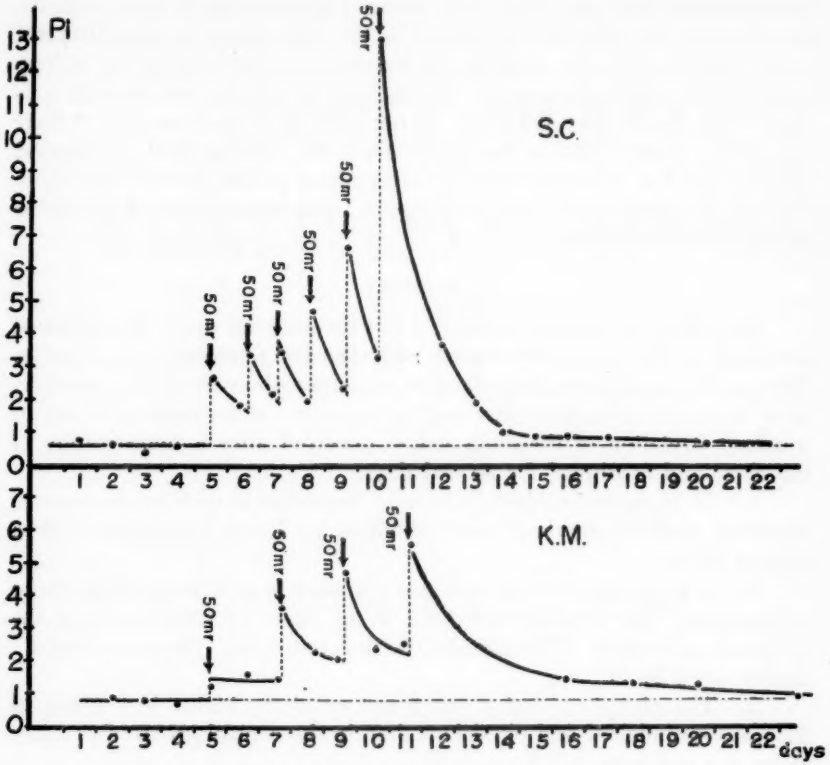

Fig. 6. Summation of effects and protracted recovery.

ported that an appreciable change in blood picture was seen in response to 0.1 mr if the dose was referred to time unit of 0.1 second. They are of the opinion that the effect is comparatively independent of the dose per day, week, etc. so far as the dose is sufficiently small, but that the effect depends greatly upon the rate (quantity per unit time). The results by those authors are in marked contrast with the experiences of others who had to give hundreds of roentgens in order to cause significant hematological changes.[2)]

If these results suggesting very high sensitivity of some hematological methods are not based on any technical failures or conscious and unconscious biases of the investigators, there must be some interpretation to reconcile the contradictory results. The present investigation seems to offer such an interpretation. As has been stated above, two kinds of effect, acute and chronic, may be distinguished, and the same may be true of

hematological changes. The acute hematological change is more sensitive to radiation, but subsides rapidly as in the experiment by Wakabayashi *et al.*, while the chronic effect is far less sensitive, but remains for a long period as in most experiments. If this view is correct, the unusual conclusion by Helde and Wahlberg is not difficult to understand; if their experiment really refers to the acute effect, the response will not depend on the total dose administered over a very long period, because the acute effect declines so rapidly that no complete summation occurs of the effects of individual exposures.

Summary

The effect of ionizing radiation was investigated upon the electrical threshold of the eye as determined with electrical phosphenes as an index. The present experiment dates back to a chance observation that an enormous increase in electrical threshold of the eye and a conspicuous rise in standard deviation of threshold values occurred after a fluorography of the chest and continued for several days.

1. In 10 normal subjects, who were beginners in such measurements, definitely positive responses were obtained to X-ray irradiation with a dose of 50 mr.

2. It was found that the standard deviation in percentage of the mean of thresholds was a better indicator of the effect of radiation than the threshold value itself. This measure was designated the "phosphene index" and denoted by Pi.

3. The phosphene index was found decidedly higher in a group of 21 X-ray-technicians and physicians than in the control group of as many university students.

4. The time-course of the effect was investigated after momentary exposure to 50 mr, and two phases, acute and chronic, were distinguished. The acute effect lasted for a few days, declining rapidly. A residual effect of several per cent of the maximum remained for over 10 days (chronic phase).

5. There was an approximately linear relationship between the maximum acute effect and the logarithm of the applied dose within a range from 1 mr to 50 mr.

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